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ENDOCRINOLOGY

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EFFECT OF DCA AND NaCl LOADING ON AMINOPTERIN INTOXICATION AND WEIGHT OF THYMUS

S. L. RABASA, FRIDA BERGMANN AND A. D. DOMÉNICO[1]

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HIGGINS and Woods (1949) and Higgins (1949) in the rat, and Dougherty and Dougherty (1950) in the mouse found that the effects of aminopterin were partly mediated by the adrenals. Lewis, Rabasa and Ferrer (1952) observed independently the same fact, and could see moreover that adrenalectomized animals were significantly less resistant to aminopterin intoxication than normals. It was shown (Lewis, Rabasa and Doménico, 1952) that a dosage of 1.25 mg./100 gm. body weight (b.w.) daily of cortisone did not afford protection, while a slight but not significantly longer survival was obtained by 0.1 mg./100 gm. (b.w.) of DCA daily. The above mentioned dosage of cortisone, given to adrenalectomized rats treated with aminopterin, brought about an atrophy of the thymus similar to that of aminopterin treated normals. It was concluded, in consequence, that the effect of this cortisone dosage was approximately the same as the one produced by the glucocorticoids of the intact rat when stimulated by the antifolic. Since this dosage failed to afford effective protection, no other ones were tested and it was assumed that no protection could probably be expected from cortisone.

In preliminary experiments DCA was administered to rats at a dosage of 0.2, 0.4 and 0.8 mg. per 100 gm. of body weight. The animals were fed the stock diet and had free access to tap water and to a 3% NaCl solution. Protection was quite constant—especially with the 0.8 mg. dosage. Upon

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[1] The authors are indebted to R. W. Linton of the American Cyanamide Co., Lederle Division, for the sample of aminopterin and to R. V. E. Humphrey of Laboratorios Organon (Argentina) for the DCA used in this experiment.

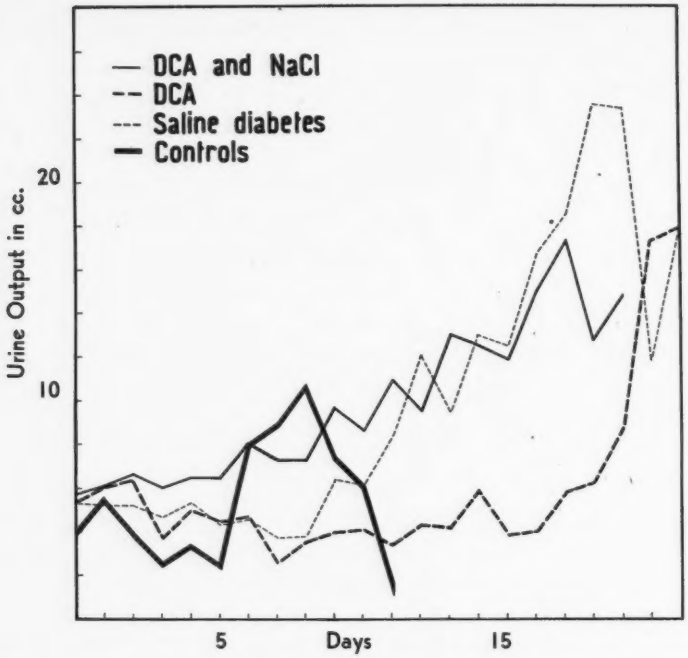

FIG. 1. Urine excretion of normal rats injected with 5 μg. per 100 gm. body weight of aminopterin daily. Each line represents a group of six rats.

receiving this higher dosage almost all these animals showed a "saline diabetes," that is, an increase of water and NaCl intake and urine output from approximately the seventh day on.

The present experiment was designed as an attempt to disclose the role played by saline diabetes, with and without the administration of DCA, in the protection against aminopterin intoxication. If the protection was due to saline diabetes, independent of DCA, then it could be attributed to the increased water exchange observed in these animals and, consequently, perhaps to an increased elimination of aminopterin which may be equivalent to a lower dosage of the drug. Conversely, if no influence is to be attributed to saline diabetes, then another mechanism should be assumed which would probably resemble more closely the normal way in which the adrenals afford protection, since this protection is not associated with a larger water exchange.

METHODS

Forty eight male albino rats weighing approximately 200 gm. were used. Twenty four of these were adrenalectomized and 48 hours afterwards both intact and operated ani-

mals were divided into four groups receiving respectively: (1) DCA[1] at a dosage of 0.8 mg./100 gm. (b.w.) daily and having free access to a 3% solution of NaCl, (2) DCA and no NaCl, (3) no DCA and no NaCl and (4) no DCA, a 3% solution of NaCl *ad lib.;* an additional quantity of water and NaCl was given this fourth group by gastric tube so as to equalize the spontaneous water and NaCl intake of the first group.

Aminopterin[1] (4-aminopteroylglutamic acid) was injected daily at a dosage of 5μg. per 100 gm. (b.w.) until death. All the animals were fed the stock diet without NaCl and had free access to tap water. Hence, groups 1 and 4 had a high water and NaCl exchange with and without DCA respectively and groups 2 and 3 had a low exchange with and without DCA. Daily records of water and NaCl intake and urine excretion were taken during the experiment. Thymi, adrenals and spleens were weighed at death.

RESULTS

Saline diabetes. An increase of urine output was obtained in the first group with DCA and NaCl *ad lib.* and the same increase was obtained when the same quantity of NaCl and water spontaneously drunk by the first group was administered to the fourth group by gastric tube without DCA (Figs. 1 and 2). The group of normals without either DCA or NaCl (group 3) (Fig. 1) showed what has been called diabetes insipidus-like syndrome

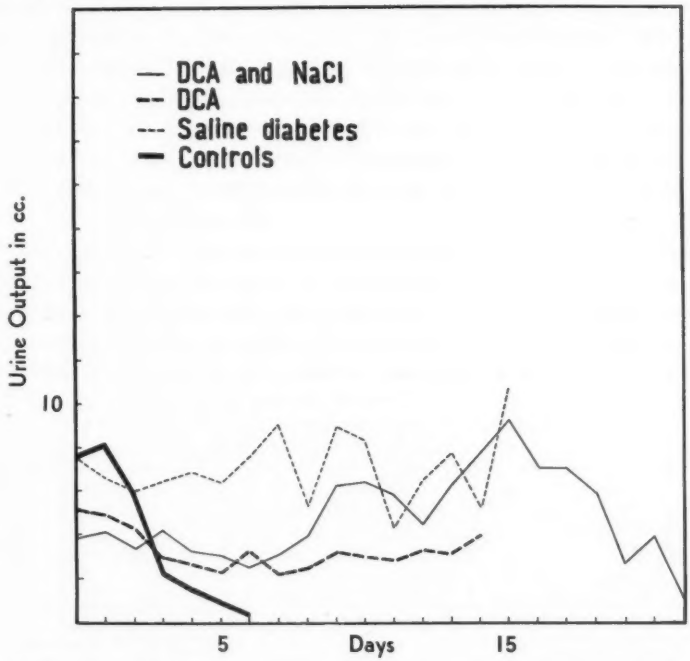

FIG. 2. Urine excretion of adrenalectomized rats injected with 5 μg. per 100 gm. body weight of aminopterin daily. Each line represents a group of six rats.

TABLE 1. MEAN LETHAL DOSE EXPRESSED AS μG. PER 100 GM. OF BODY WEIGHT OF AMINOPTERIN INJECTED UNTIL DEATH, AT A DOSE OF 5 μG. DAILY

Rats	DCA 0.8 mg. per 100 gm. daily		No DCA	
	NaCl ad lib.	No NaCl	NaCl and water ad lib. and by stomach tube	No NaCl
Normal	89.2	79.2	93.8	45.8
Adrenalectomized	70.0	65.8	60.8	35.8

(Lewis, Rabasa and Ferrer, 1952), i.e., an increase of water intake and output of sugar-free urine toward the end of the intoxication period.

As previously reported (ibid.) adrenalectomized rats without injected adrenal hormones do not show diabetes insipidus (Fig. 2). DCA injected normal animals without NaCl (group 2) had the lowest urine excretion, but, as in all normal groups, it was increased at the end of the intoxication period.

Adrenalectomized rats showed the impairment of diuresis described by many authors (Gaunt, Birnie and Eversole, 1949), but, although they could not reach the level of excretion of normals, DCA and/or saline treated groups had a larger output (as in the normal groups). Although less clearly than in normals, two levels of excretion were apparent whether NaCl was given or not. As reported previously (Lewis, Rabasa and Doménico, 1952), adrenalectomized rats receiving DCA and NaCl (Fig. 2) also exhibited an increase of diuresis, similar to that of normals, toward the end of the experiment.

Survival. Both DCA and saline diabetes afforded protection against aminopterin intoxication (Table 1). Considered independently (see Analysis of Variance, Table 2) the rats with saline diabetes showed more resistance than those receiving DCA. Maximum protection was achieved, however, in salt loaded normals without DCA. DCA and saline diabetes

TABLE 2. ANALYSIS OF VARIANCE, DATA OF TABLE 1
$F_{0.05} = 4.08$, $F_{0.01} = 7.31$

Sources	D. of F.	Sx^2	F
Saline diabetes	1	6 302.00	12.26
Adrenals	1	4 799.00	9.34
DCA	1	3 008.00	5.85
Interactions			
DCA-Saline diab.	1	3 008.00	5.85
Adren.-Sal. diab.	1	833.00	
Adr.-Sal. diab.-DCA	1	351.83	
Adren.-DCA	1	168.50	
Error	40	20 559.00	

TABLE 3. WEIGHT OF THYMUS (MG.) PER 100 GM. OF BODY WEIGHT

Rats	DCA, 0.8 mg. per 100 gm. daily		No DCA	
	NaCl ad lib.	No NaCl	NaCl and water ad lib. and by stomach tube	No NaCl
Normal	28.4	19.2	19.3	21.2
Adrenalectomized	75.0	55.0	101.3	76.2

did not sum their effects. This interaction (DCA-NaCl) is statistically significant ($P < 0.05$). The same proportionate scale of reactions is true in both adrenalectomized and normal rats. However, adrenalectomized rats have less resistance than normals and this difference is highly significant ($P < 0.01$).

The interaction of the adrenals with saline diabetes is not significant; neverthelesss the sum of its squares is the largest among the remaining non-significants. This fact is due to the negligible survival difference existing between normal and adrenalectomized rats not receiving both NaCl and DCA. However, a significant difference is obtained when these groups are given NaCl by stomach tube, because of the protective effects of saline diabetes. It seems that NaCl is necesasary for the adrenal to protect and, reciprocally, saline diabetes protection is better exerted when the adrenals are present. DCA, on the contrary, acted almost the same with and without either NaCl or the adrenals. When adrenals and/or NaCl were present the effect of DCA was larger, but the difference was not significant.

Thymus. The thymus was larger in rats showing saline diabetes and, obviously, also in adrenalectomized rats. DCA decreased its size but not to a significant degree (Tables 3 and 4).

There is a significant interaction of DCA and the adrenals: DCA-injected intact rats given NaCl had slightly larger thymi while adrenalectomized DCA-injected rats, with and without NaCl, showed smaller thymi than when no DCA was employed.

TABLE 4. ANALYSIS OF VARIANCE. DATA OF TABLE 3. $F_{0.05} = 4.09$ $F_{0.01} = 7.33$

Sources	D. of F.	Sx^2	F
Adrenals	1	36 124.2	108.80
Saline diabetes	1	2 051.5	6.18
DCA	1	1 215.5	
Interactions			
Adrenals-DCA	1	2 224.5	6.70
Adrenals-saline diab.	1	1 077.2	
Adrenals-DCA-sal. diab.	1	198.5	
DCA-Saline diab.	1	27.2	
Error	39	12 947.3	

Adrenalectomy increased, although not significantly, the difference between the weight of the thymus of rats with and without saline diabetes.

Spleen and adrenals. No significant differences were found in the weights of the spleens and adrenals of these groups.

DISCUSSION

As was expected, it was not possible to obtain in the adrenalectomized rats a rate of urine excretion equal to that of normals. Nevertheless, those with NaCl had a higher output than adrenalectomized without NaCl from the beginning of the experiment, and those with DCA and NaCl increased their excretion from the seventh day on (Fig. 2) whereas the groups without NaCl had a persistently low elimination.

There is a striking difference between the urine output of normals without either DCA or NaCl and that of their adrenalectomized controls. The latter showed a very small and declining excretion while the former developed a diabetes insipidus-like sysndrome (Lewis, Rabasa and Ferrer, 1952). Not withstanding, their survival periods were not significantly different. Moreover, DCA-treated rats (both normal and adrenalectomized) without NaCl had longer survival than the preceding groups (those without either DCA or NaCl) in spite of being definitely oliguric, and no significant survival increase could be obtained by adding NaCl, although this increased urine output.

It seems, in consequence, that the longer survival observed in the groups treated with both DCA and/or NaCl was not due to the increase of excretion, although, actually, a positive but not significant correlation ($r = 0.556$, $r_{0.05} = 0.71$) exists between rate of excretion and survival.

As the lack of NaCl reduces the survival of normals to a point not significantly different from that of adrenalectomized rats also lacking NaCl, it seems safe to conclude that this salt is necessary for the adrenal to protect. In fact, when a saline diabetes (without DCA) is induced both normal and operated rats are protected, but there is a significantly longer survival in normals. DCA, contrary to the adrenal, seems to need less NaCl to act. In the former groups (without DCA) the combination of saline diabetes and adrenals afforded a greater protection than the summation of their effects when taken independently, while the protection of DCA and saline diabetes were not additive. It could be assumed that DCA acts through its NaCl retaining effect exerted on the amount of this salt present in the animal at the onset of the experiment, because they had, up to this moment, a free access to a NaCl solution. Accordingly, those rats deprived of NaCl during the experiment might have a sufficient load of salt for DCA to act on.

The effect of saline diabetes is probably due, therefore, to NaCl. Experiments now in progress have been designed to clarify this point. It seems safe to assume that DCA and NaCl probably act through the same mechanism, while the adrenals themselves, although needing NaCl to operate, have a more complex mechanism allowing them to afford a greater protection.

The protective effect of the adrenals, DCA and NaCl on aminopterin intoxication and the ineffectiveness of cortisone on it, parallel well with the facts observed by Ellinger, 1947 and 1952, and Ellinger and Blagg, 1952, in x-irradiated animals where also adrenals, DCA and normal saline protected, while no effect seemed ascribable, in this connection, to cortisone. Straube, Patt, Tyree and Smith (1949), on the contrary, failed to find DCA protection, and Mirand, Reinhard and Goltz (1952) obtained similar protective effects with DCA and cortisone.

The thymi of the animals with saline diabetes, excepting normals without DCA, were significantly larger in spite of their longer survival, which means that a larger quantity of aminopterin was administered and, accordingly, a greater atrophy should be expected. DCA treated animals had larger thymi if normal, and smaller if adrenalectomized. This significant interaction might be explained as follows: DCA has, at this dosage, a thymolytic effect which can be measured in adrenalectomized rats. In normals, on the contrary, its restraining effect upon the adrenals, slightly diminishing the production of glucocorticoids, causes less atrophy of thymus because a powerful thymolytic hormone is replaced by a weaker one. This opinion is partly substantiated by the suggested but not statistically significant difference in the weights of the adrenals observed in DCA-treated animals as compared with those not receiving this steroid.

SUMMARY

The survival of animals treated with toxic doses of aminopterin is increased by NaCl-loading or DCA and decreased by adrenalectomy.

NaCl loading protects both normal and adrenalectomized rats, but its effect is significantly greater when the adrenals are present.

DCA provides protection without added NaCl; no significant difference exists between normal and adrenalectomized rats injected with DCA.

The thymi of adrenalectomized rats, and those of NaCl-loaded ones are larger than their controls ($P < 0.01$ and $P < 0.05$ respectively).

The atrophy of thymus is less in DCA-treated normals than in those not receiving DCA.

DCA induces a slight atrophy of thymus in adrenalectomized rats ($P < 0.05$).

Acknowledgment

We wish to express our gratitude to Mr. Donald W. Sutherland, Director of the United States-Argentine bi-national Cultural Center in Rosario, for helpful collaboration in the preparation of the manuscript.

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THE SECRETION OF AN ANTIDIURETIC SUBSTANCE INTO THE CIRCULATION OF RATS EXPOSED TO NOXIOUS STIMULI[1,2]

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with the technical assistance of ROBERT JINKS

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EXPOSURE to a physical or emotional stimulus which is noxious to animal or man results in an inhibition of the diuresis induced by the ingestion of water (Pickford, 1945; Verney, 1947; Kelsall 1949). This antidiuretic response to a stressful stimulus is diminished in the absence of the neurohypophysis (O'Conner and Verney, 1942). Consequently, it has been postulated that the antidiuretic response is dependent upon the activation of the neurohypophysis and the release of an "anti-diuretic hormone" into the circulation.

Direct evidence that an increase in the antidiuretic activity of the blood occurs when an animal is exposed to a noxious stimulus is not available. With the development of a relatively simple, sensitive and precise procedure for the assay of antidiuretic substances (ADS) in the blood plasma (Stein, Jinks, and Mirsky, 1952), it became possible to determine the activity of the plasma from rats exposed to noxious stimuli.

METHOD

Male albino rats of the Carworth strain, weighing from 140 to 250 grams were maintained on Purina laboratory chow until sacrified for the collection of blood. Rats which were used for evaluating the effect of various stimuli on the rate of excretion of a water load were fasted for approximately 16 hours during which time they were permitted access to water.

Blood was collected in oxalated tubes after decapitation and the plasma was separated rapidly by centrifugation. In order to obtain an adequate quantity for assay, equal amounts of the plasma obtained from four to six rats were pooled and assayed within one to two hours by the procedure described by Stein, Jinks and Mirsky (1952).

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[1] Presented in part before the XIX International Physiological Congress, Montreal, September, 1953.

[2] Aided in part by grant from the Eli Lilly Company.

[3] U. S. Public Health Service Research Fellow of the National Institute of Mental Health.

The assay rats were kept in individual metabolism cages and given 5 ml. of warm 0.2% NaCl per 100 gm. body weight by stomach tube. One hour later, the gavage was repeated, and twenty minutes after this gavage, 1 ml. of plasma per 100 gm. body weight or a similar volume of a solution of Pitressin in isotonic saline was injected intraperitoneally. The animals were handled and prodded every twenty minutes after the second gavage and the volume of urine excreted at the 20 and 60 minute intervals was recorded. The response to the intraperitoneal injection was expressed as the reciprocal of the ratio of the volume of urine excreted by 60 minutes to that excreted by 20 minutes after the second gavage. At the outset, the antidiuretic activity of the plasma was estimated by the two-dose factorial design of Bliss (1944) with four groups of five rats; two groups were injected with 0.3 and 0.6 mU Pitressin[4] per 100 gm. body weight respectively, and two groups with 1 ml. 50% diluted plasma and 1 ml. intact plasma per 100 gm. body weight respectively. When it became apparent that the slope of the dose-response curve for plasma was essentially the same as that for Pitressin, only one group of rats was injected with plasma and two groups with Pitressin. The plasma ADS values, expressed as mU per 100 ml. equivalents of Pitressin, were then computed as follows: Let X be the quantity of ADS in terms of mU Pitressin per ml. of plasma and Y be the mean response of the group of five rats treated with the plasma, then $\log X = (Y + b\bar{x} - \bar{y})/b$, where b is the slope of the log dose-response curve obtained in the two groups injected with Pitressin, $\bar{x}$, the mean of all the dosages of the standard expressed logarithmically and $\bar{y}$ the mean of all the responses of the rats treated with the two dosages of standard.

The antidiuretic response to various stimuli was determined by preparing rats as for the assay and exposing them to the particular stimulus at 20 minutes after the second gavage.

Pain, noise, a strange environment and the injection of histamine were employed as stimuli for evoking an antidiuretic response. For comparison, the effect of a "physiological" stimulus, dehydration, was determined by depriving rats of water for 24 hours. Pain was produced by exposing each rat in a small metabolism cage to a two-minute period of repetitive mild electric shocks to the feet via a grid which made up the floor of the cage. Noise was produced by means of a siren (Federal Siren, Type C6, 6 volts) to which rats in individual cages were exposed for five minutes. The strange environment consisted of a two compartment conditioning box, into which the rats were placed individually for variable periods of time. The two ends and the top of the apparatus were white, one side consisted of white milk glass through which illumination was provided and the remaining side consisted of a black one-way vision screen. The response to histamine was determined at intervals after the intraperitoneal injection of 1 mg. histamine hydrochloride in 0.2 ml. normal saline per 100 gm. of body weight.

RESULTS

Response to Water Deprivation

The concentration of ADS in the plasma of 97 rats permitted free access to water was 18.4 ± 1.4 mU per 100 ml. while that of 86 rats deprived of water for 24 hours was 29.7 ± 2.3 mU per 100 ml. (Fig. 1). Although the difference is relatively small, it is statistically highly significant ($P < 0.001$).

[4] We are indebted to Dr. D. A. McGinty of the Parke Davis Company for a supply of Pitressin which assayed to 40 U.S.P. Units per mg.

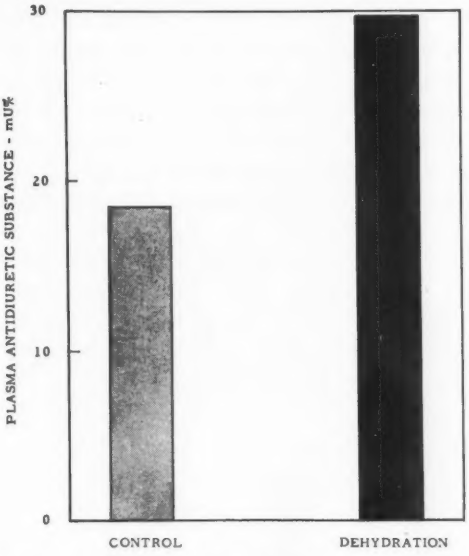

FIG. 1. The effect of water deprivation for 24 hours on the antidiuretic activity of the plasma of rats.

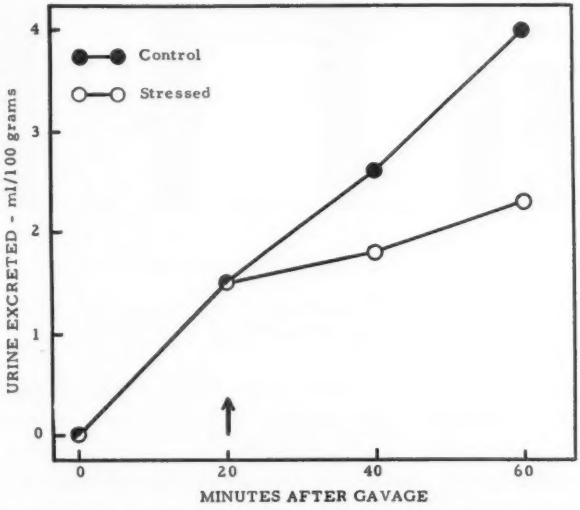

FIG. 2. The antidiuretic response of rats to painful stimuli applied to the feet. Five ml. of 0.2% NaCl per 100 grams body weight were given at one hour before and again at 0 time. At the time designated (↑), one group of 26 rats (○—○) were exposed for two minutes to repetitive mild electric shocks to the feet while the other group of 42 rats was not disturbed (●—●).

Response to Painful Stimuli

Repetitive mild electric shocks to the feet for two minutes produced obvious painful responses in the rats. The effect of such painful stimuli on the diuresis induced by a water load is illustrated in Figure 2 which depicts the means of the responses of 42 rats which served as controls and of 26 rats which were exposed to the painful stimuli at the indicated time. It is evident that the exposure to the noxious stimuli resulted in a marked reduction

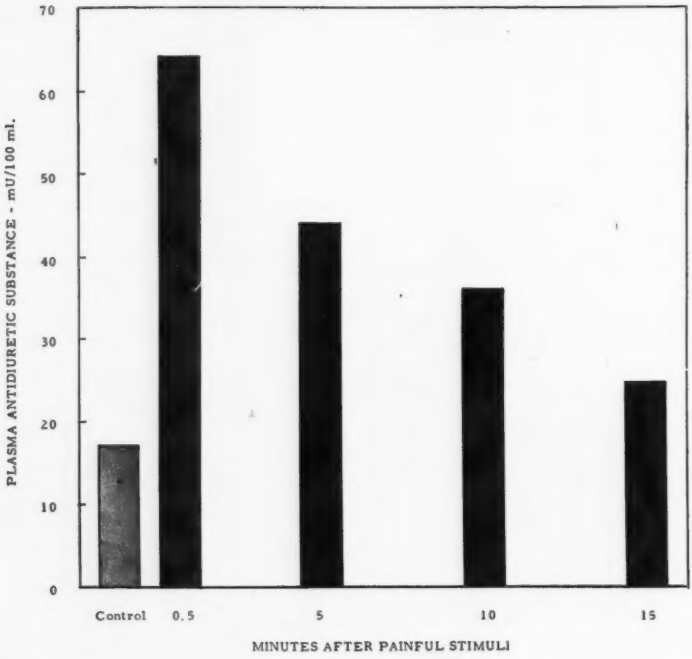

FIG. 3. The antidiuretic activity of the plasma of rats exposed for two minutes to painful stimuli applied to the feet. (See text.)

in the volume of urine excreted by the rats. Both groups of rats excreted urine at the rate of 0.076 ml. per 100 gm. of body weight during the first twenty minutes. Thereafter, the untreated rats continued to excrete at essentially the same rate during the second (0.055 ml. per minute) and third (0.068 ml. per minute) 20-minute intervals, while the rats exposed to the painful stimuli excreted at the rate of 0.007 ml. per minute and 0.03 ml. per minute during the same intervals. The differences between the rates of excretion by the 40 and 60 minute intervals were statistically highly significant ($P < 0.001$).

The effect of exposure to the painful stimuli on the concentration of ADS in the plasma is illustrated in Figure 3. Whereas the concentration of ADS in the plasma from 97 untreated rats was equivalent to 18.4 ± 1.4 mU of Pitressin per 100 ml., that of 41 rats was 64.3 ± 6.0 per 100 ml. within one-half minute after exposure to the painful stimuli. Five minutes after such exposure, the ADS titer of the plasma from 41 rats was 43.9 ± 7.3 mU per 100 ml., in 10 minutes, that of 35 rats was 36.2 ± 5.2, and in 15 minutes, that of 17 rats was 24.8 ± 3.9 mU. The differences between the values obtained in normal rats and in animals bled 0.5, 5.0 and 10 minutes after exposure to the painful stimuli were statistically highly significant ($P < 0.001$). The data obtained when the Bliss design was employed in the assay of the plasma from another set of rats treated in a similar manner are summarized in Table 1, and reveal essentially the same response to the painful stimuli.

TABLE 1. EFFECT OF PAINFUL STIMULI ON PLASMA ADS OF NORMAL RATS

$$\frac{0.6 \text{ mU Pitressin}}{0.3 \text{ mU Pitressin}} = \frac{1 \text{ ml. Plasma}}{0.5 \text{ ml. Plasma}} = 2$$

(Log Dose—Response)

Minutes after noxious stimulus	N[1]	Slope b	Index of precision λ	Significance of difference between slopes t[2]	Potency ratio M ± S.E. %	Pitressin equivalent M ± S.E. mU/100 ml. plasma
Control	5	0.5697	0.1675	1.21	37.59 ± 11.23	22.6 ± 6.8
Control	5	0.4060	0.1128	3.23	37.44 ± 7.55	22.5 ± 4.5
0.5	5	0.9370	0.1124	1.25	94.30 ± 10.93	56.6 ± 6.6
0.5	5	0.5543	0.2255	1.91	124.60 ± 30.32	74.7 ± 18.2
5.0	5	0.5020	0.1878	0.25	54.10 ± 13.96	32.5 ± 8.4
5.0	5	0.4773	0.1714	1.22	45.07 ± 12.13	27.1 ± 7.3
10.0	5	0.4070	0.1098	0.98	64.07 ± 8.60	38.5 ± 5.2
10.0	5	0.2753	0.4264	0.39	50.00 ± 31.08	30.0 ± 18.7
15.0	5	0.3587	0.2478	1.21	43.71 ± 17.38	26.2 ± 10.4

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$

[1] Animals used at each dose level.
[2] 5% level of significance when $t = 2.179$.

Response to Noise

Exposure to the noise produced by a siren for 4 minutes induced no significant behavioral response except for an increase in the grooming activities of the rats. The influence of the noise on the antidiuretic activity of the plasma is illustrated in Figure 4. The statistically highly significant difference ($P < 0.001$) between the plasma ADS concentrations of the 97 untreated rats used for controls (18.4 ± 1.4 mU per 100 ml.) and the 44 rats

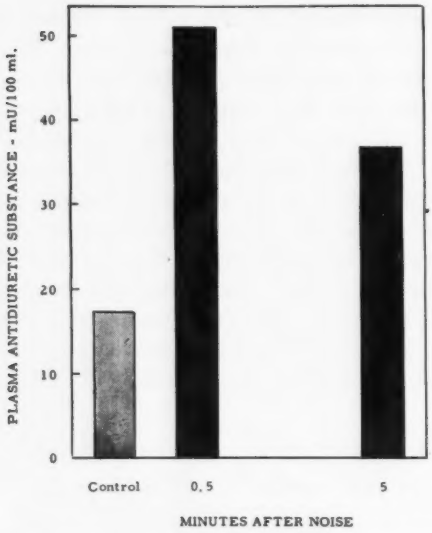

FIG. 4. The antidiuretic activity of the plasma of rats exposed for five minutes to the noise produced by a siren. (See text.)

bled within 30 seconds after exposure to noise (50.9 ± 3.5 mU per 100 ml.) is indicated also by the data obtained with the Bliss design in another group of rats (Table 2). The mean ADS concentration of the plasma from

TABLE 2. EFFECT OF NOISE ON PLASMA ADS OF NORMAL RATS

$$\frac{0.6 \text{ mU Pitressin}}{0.3 \text{ mU Pitressin}} = \frac{1 \text{ ml. Plasma}}{0.5 \text{ ml. Plasma}} = 2$$

(Log Dose—Response)

Treatment	N[1]	Slope b	Index of Precision λ	Significance of Difference Between Slopes t[2]	Potency Ratio M ±S.E. %	Pitressin Equivalent M ±S.E. mU/100 ml. Plasma
Control	5	0.5697	0.1675	1.21	37.59 ±11.23	22.6 ± 6.8
Control	5	0.4060	0.1128	3.23	37.44 ± 7.55	22.5 ± 4.5
Noise	5	0.8873	0.0964	1.39	80.26 ± 4.53	48.2 ± 5.0
Noise	5	0.3880	0.3562	0.28	97.29 ±25.67	58.4 ±21.4
Noise	5	0.6440	0.1217	2.68	106.50 ±13.40	63.9 ± 8.0

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$

[1] Animals used at each dose level.
[2] 5% level of significance when $t = 2.179$.

two sets of rats 5 minutes after exposure to the siren was 36.8 mU per 100 ml.

Response to a Strange Environment

Introduction of the rats into the strange environment represented by the two-compartment conditioning box produced no overt behavioral responses other than an increase in grooming activities. The effect of such exposure to the concentration of ADS in the plasma is depicted in Figure 5. No statistically significant difference was noted ($P>0.2$) between the

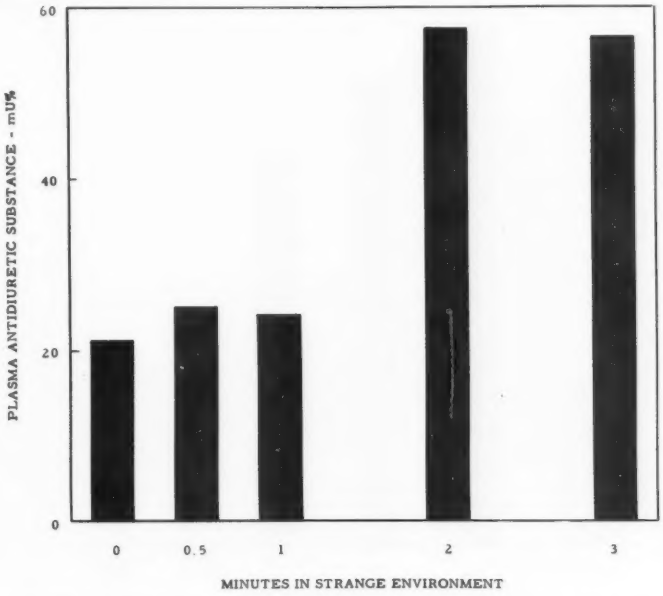

FIG. 5. The antidiuretic activity of the plasma of rats exposed to a strange environment. (See text.)

plasma ADS concentration of five groups of rats which were handled but not exposed to the box (21.1 ± 1.5 mU per 100 ml.), of five groups of rats which were in the box for 30 seconds (25.1 ± 6.3 mU per 100 ml.) and of five groups of rats which were in the box for one minute (23.9 ± 4.9 mU per 100 ml.). The values obtained after the rats were in the box for two minutes (57.3 ± 6.2 mU per 100 ml.) and three minutes (56 ± 9.4 mU per 100 ml.) differed significantly from those of rats exposed to the strange environment for shorter intervals ($P<0.001$). The ADS titer of the plasma of a single group of 5 rats exposed to the interior of the box for 10 minutes was similar to that of the rats exposed for 2 or 3 minutes.

Response to Histamine

The overt reaction of rats injected with one mg. histamine hydrochloride per 100 gm. of body weight differed in no respect from that of rats injected with normal saline. Since the concentration of ADS in the plasma of groups of rats bled at various intervals after the injection of normal saline remained unchanged ($P>0.2$), all control values were pooled and the mean depicted as such in Figure 6. This figure illustrates also the mean response

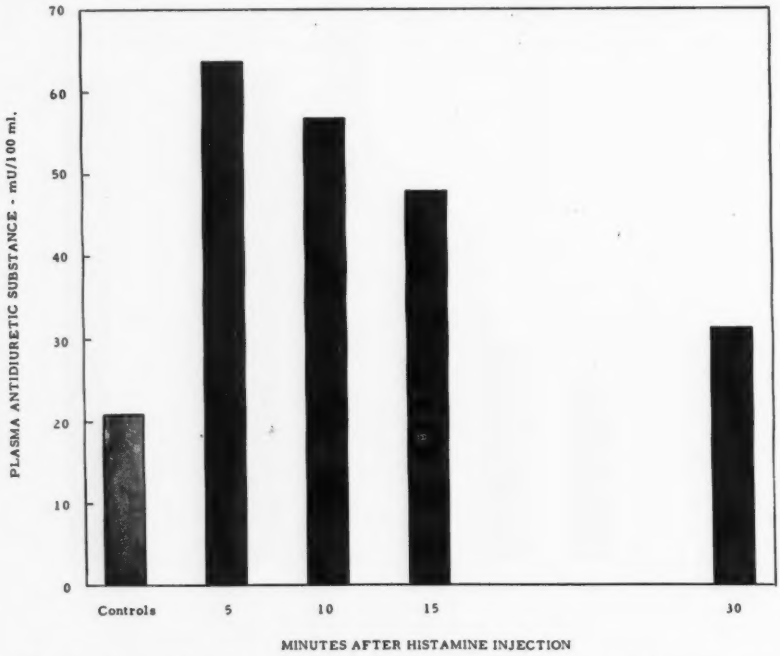

FIG. 6. The antidiuretic acitvity of the plasma of rats given an intraperitoneal injection of Histamine. (See text.)

of five groups of 5 rats bled at 5, 10, 15 and 30 minutes after the injection of histamine. Whereas the ADS titer of the saline injected animals was 20.9 ± 2.2 mU per 100 ml., that of the histamine treated rats was 63.6 ± 7.0 mU per 100 ml. in 5 minutes, 56.7 ± 6.4 mU per 100 ml. in 10 minutes, 47.7 ± 4.2 mU per 100 ml. in 15 minutes, and 31.4 ± 4.3 mU per 100 ml. in 30 minutes. The difference between the control group and each of the groups bled 5, 10 and 15 minutes after the injection of histamine was statistically highly significant ($P<0.001$). The group bled 30 minutes after the injection of histamine differed little from the control ($P=0.05$–0.1).

Response of Hypophysectomized Rats to Injected Plasma

The assay procedure employed herein is dependent upon the assumption that the antidiuretic response of the normal hydrated rat is due to the presence of an antidiuretic substance in the plasma and not to the release of an "antidiuretic hormone" by the assay animal's own neurohypophysis. To test this assumption, completely hypophysectomized rats, 5–10 day after operation, were given an intraperitoneal injection of cortisone and two hours

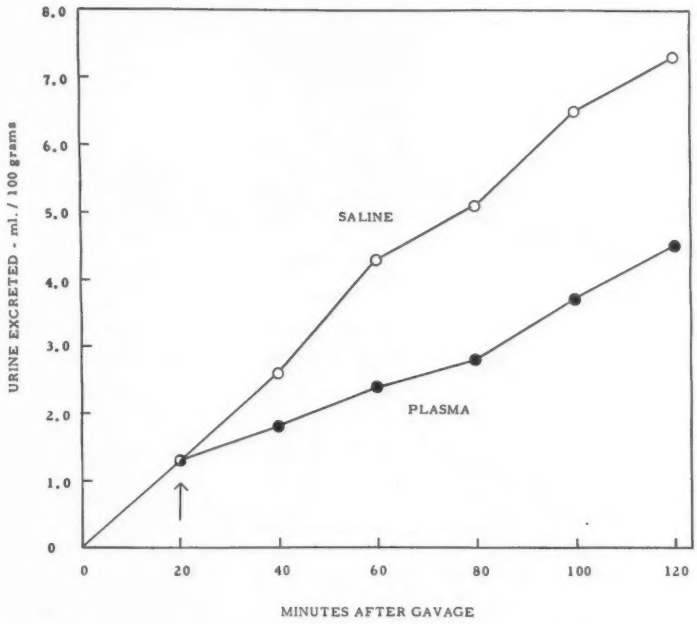

FIG. 7. The antidiuretic response of cortisone treated hypophysectomized rats to the intraperitoneal injection of plasma from rats exposed to painful stimuli. (See text.)

later were given 0.2 per cent NaCl and treated as the normal rats used for assay. Although hypophysectomized rats have a markedly diminished diuretic response to a water load, a partial restitution occurs with the administration of adrenal cortical hormones (Boss and Osborn, 1950). Figure 7 depicts the mean response of 5 such hypophysectomized rats to the intraperitoneal injection of saline and of plasma from rats previously exposed to painful stimuli. It is evident that the hypophysectomized rats responded to the injection of the plasma with a significant reduction in the rate of urine flow.

Effect of Plasma on the Diuresis of Dogs

There is reason to suspect that the antidiuretic response of rats to the intraperitoneal injection of plasma, serum or urine may be peculiar to that species and differ from the response of dogs (Ames, Moore and VanDyke, 1950) or men (Lewis, 1953). Consequently, the effect of the intravenous injection of various amounts of plasma on the diuresis of the hydrated bitch was studied. Figure 8 illustrates the typical antidiuretic response of

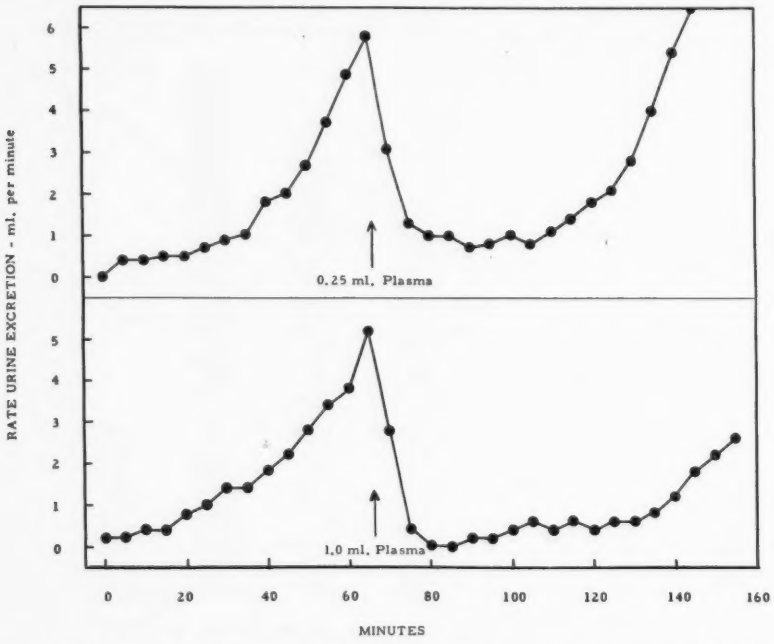

FIG. 8. The antidiuretic response of hydrated dogs to the intravenous injection of plasma from normal rats. Trained dogs were given water by stomach tube at 0 time and the urine secreted was collected through a catheter and recorded at five minute intervals.

such hydrated dogs to the intravenous injection of plasma from normal untreated rats.

Since an antidiuretic response to the injection of plasma may be due to a decrease in glomerular filtration rather than to an increased reabsorption of water, the effect of plasma on the excretion of creatinine by the hydrated dog was studied. In accord with the observations of Hare, Melville, Chambers and Hare (1945) on the effect of Pitressin, the injection of normal rat plasma resulted in a concomitant decrease in flow and increase in endogenous creatinine concentration of the urine (Fig. 9).

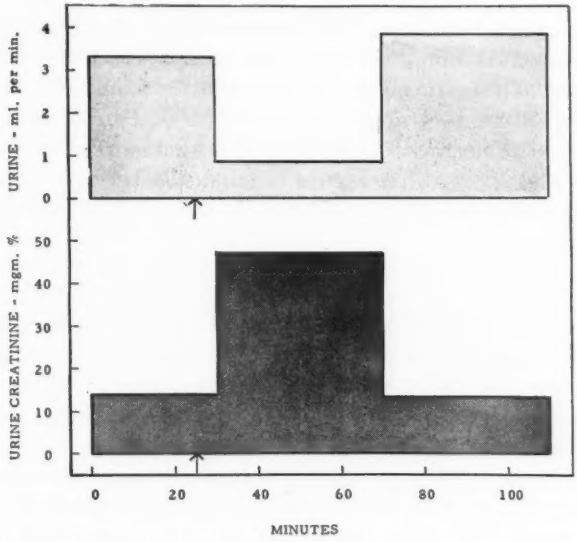

FIG. 9. The effect of the intravenous injection of plasma from normal rats on the rate of urine formation and the concentration of urine creatinine of the hydrated dog.

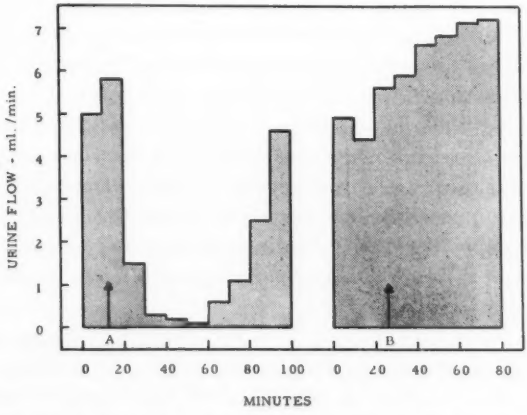

FIG. 10. The effect of treatment with thioglycollate on the antidiuretic activity of plasma from rats exposed to painful stimuli. Hydrated dog injected intravenously with saline diluted plasma at "A" and with similar concentration of thioglycollate treated plasma at "B."

Effect of Thioglycollate on the Plasma ADS

Ames, Moore and VanDyke (1950) demonstrated that the antidiuretic activity of Pitressin is diminished after treatment with thioglycollate. Accordingly, plasma from rats exposed to painful stimuli was mixed with sodium thioglycollate to a final concentration of 0.02M at pH 7.5 for various periods of time while an aliquot of the same plasma was diluted with an equal volume of saline. Figure 10 illustrates the inactivating effect of such treatment on the antidiuretic activity of the plasma as measured in the hydrated dog.

DISCUSSION

The nature of the substance or substances responsible for the antidiuretic activity of the plasma is unknown. Its effectiveness on the diuresis of hydrated dogs as well as that of rats distinguishes plasma ADS from some factor which is antidiuretic only on intraperitoneal injection in rats (Ralli, Raisz, Leslie, Dumm and Laken, 1950). Its effectiveness on intraperitoneal injection in cortisone treated hypophysectomized rats distinguishes plasma from such factors as ferritin which act through stimulation of the assay animal's own neurohypophysis (Baez, Mazur and Shorr, 1952). Its effectiveness in producing a concomitant decrease in the rate of flow and increase in the concentration of endogenous creatinine in the urine of the hydrated dog distinguishes plasma ADS from factors which act on glomerular filtration rather than on tubular reabsorption.[5] Its inactivation by sodium thioglycollate suggests that plasma ADS resembles the antidiuretic hormone of the neurohypophysis as does also the absence of a statistically significant difference between the slope of the dose-response curve of plasma ADS and that of Pitressin ($P = 0.1$).

The relatively significant quantities of ADS found in the plasma of untreated normal rats suggests the presence of some factor other than, or in addition to, the antidiuretic hormone, or may reflect the quantitative limitations of the assay procedure. On the other hand, the trauma involved in the process of decapitating and bleeding the rats may be responsible for the release of some ADS into the circulation. Since the same procedures

[5] In accord with the suggestion of the reviewers of this manuscript, the creatinine concentration of the urine excreted by the rats used in the assay was determined before and after the intraperitoneal injection of plasma from rats exposed to painful stimuli. In a typical experiment, the mean rate of urine excretion during the twenty minutes before the injection was 0.07 ml. per 100 gm. per minute and the mean creatinine concentration of the urine was 4.4 mg. per 100 ml. During the forty minutes after the injection, the mean rate of urine excretion fell to 0.03 ml. per 100 gm. per minute while the concentration of creatinine rose to 8.7 mg. per 100 ml. Consequently, the ADS of the plasma produces an increase in the tubular reabsorption of water in the assay rats.

were utilized for both control and experimental groups, the differences can be attributed to the experimental variable.

Dehydration may be regarded as a "physiological" stimulus to the secretion of the antidiuretic hormone since it results in a depletion of the antidiuretic activity of the neurohypophysis (Kovacs and Bachrach, 1951; Hild and Zetler, 1953), and an increase in the activity of serum (Jenkins and Birnie, 1949) and urine (Gilman and Goodman, 1937; Boylston and Ivy, 1938; Martin, Herrlich and Fazekas, 1939; Hare, Hickey and Hare, 1941; Ham and Landis, 1942). In accord with the above, a small but significant increase in the antidiuretic activity of the plasma is found in rats which have been deprived of water for 24 hours.

Irrespective of the precise nature of the ADS, an unequivocal increase occurs in the plasma of rats exposed to painful stimuli, noise, a strange environment or the injection of histamine. This observation offers an explanation for the marked diminution in the rate of urine excretion which occurs in rats exposed to such varied stimuli as exercise (Karady, Browne and Selye, 1938), surgical trauma (Howlett and Browne, 1940), mechanical shaking (Schreiber, 1950), flashing lights (Boyd, Lee and Stevens, 1943), histamine (Howlett and Browne, 1940), epinephrine (Karady, Browne and Selye, 1938), formaldehyde (Kovacs and Bachrach, 1951). In all these instances, the antidiuresis may be attributed to the release of ADS into the circulation.

The rapidity with which the antidiuretic activity of the plasma is increased after exposure to the various stimuli is very striking. Even with a relatively subtle stimulus, such as the strange environment, a maximum plasma ADS concentration is attained within two minutes. With the cessation of the stimuli, the ADS concentration decreases at a relatively slower rate and is almost back to the pre-exposure level within ten to fifteen minutes. The rapid increase and the more gradual decrease suggest that ADS is rapidly secreted into the circulation in response to the stimuli and then is gradually removed or destroyed.

The present study offers no explanation of the mechanism whereby noxious stimuli result in a release of ADS into the circulation. Whereas it can be postulated that painful stimuli to the feet of the rat may produce some local metabolic agent, such an inference is improbable with respect to noise or to the emotional reaction of the rat to a strange environment. It is more likely that the different kinds of noxious stimuli are effective through activation of some common mechanism with a subsequent explosive release of ADS into the circulation.

Cannon's extensive observations (1929) and the more recent studies by Long (1952) suggest that noxious stimuli may be effective through activation of the sympathetic nervous system and the secretion of epinephrine

into the circulation or the local release of nor-epinephrine. Such a common mechanism, however, cannot be responsible for the release of ADS into the circulation since the administration of epinephrine or nor-epinephrine produces diuresis in normal rats (Drill and Bristol, 1951) and may inhibit the antidiuresis resulting from exposure to noxious stimuli (Verney, 1947). Further, even after denervation of the kidneys and adrenals and removal of the abdominal sympathetic chains, dogs show an antidiuretic response to noxious stimuli (Rydin and Verney, 1938).

Activation of the hypothalamus is a common response to the different specific reactions induced by a variety of noxious stimuli (Gellhorn, 1953). Such activation may release large quantities of ADS into the circulation since a marked antidiuretic response results from electrical stimulation of the supraoptico-hypophyseal tract of the hypothalamus (Harris, 1948) while degeneration of the supraoptic and paraventricular nuclei or of the supraoptico-hypophyseal tract results in diabetes insipidus (Fisher, Ingram and Ranson, 1938). Accordingly, it may be postulated that the specific reactions induced by sensory stimuli (pain, noise), systemic stimuli (histamine) and by emotional stimuli (strange environment) activate the hypothalamus with a resultant release of the antidiuretic hormone from the neurohypophysis as is postulated by some (Verney, 1947; Pickford, 1952) or from the supraoptic, paraventricular and other parts of the hypothalamus as is suggested by the observations of others (Bargmann and Scharrer, 1951; Kovacs and Bachrach, 1951; Hild and Zetler, 1952, 1953; Leveque and Scharrer, 1953).

Irrespective of the precise nature and source of the ADS secreted in response to exposure to noxious stimuli, it may well be one of the earliest of the common responses to such exposure. Further, the change in the concentration of the ADS in the plasma may serve as a quantitative index of hypothalamico-neurohypophyseal activation.

SUMMARY AND CONCLUSIONS

Plasma from normal rats can produce an antidiuretic response in the hydrated rat or dog. This response is attributed to some substance (or substances) which is similar to the antidiuretic hormone. In support is the small but significant increase in plasma ADS which occurs after deprivation of water for 24 hours.

Exposure for two minutes to painful stimuli produced by repetitive mild electric shocks applied to the feet of rats results in an inhibition of the diuretic response to a water load and in a rapid, marked increase in the antidiuretic activity of the plasma. Exposure for five minutes to the noise produced by a siren, for two minutes to a strange environment or for five minutes to the action of histamine results in a marked increase in the anti-

diuretic activity of the plasma. With the cessation of the stimuli, there is a relatively rapid reduction in the activity so that the pre-stimulation level is attained within 10 to 15 minutes.

It is postulated that the specific reactions induced by sensory stimuli (pain, noise), systemic stimuli (histamine) or emotional stimuli (strange environment) activate the hypothalamus with a resultant release of an antidiuretic substance into the circulation.

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BIOPHYSICAL AND CHEMICAL INVESTIGATION ON BONE TISSUE IN EXPERIMENTAL HYPERPARATHYROIDISM[1]

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INTRODUCTION

THERE have been considerable differences of opinion concerning the mode of action of the parathyroid hormone on bone tissue. The discussion of these problems have followed two main lines, one school holding that the effects of the hormone are secondary to an increased excretion of phosphate in the urine and the consequent lowering of the phosphate level in the blood (Albright and Ellsworth, 1929) and the other school maintaining that the parathyroid hormone exerts a direct action which causes bone resorption (Thomson and Collip, 1932). Barnicot (1948) and Chang (1950), working with grafts of parathyroid, have shown that these grafts caused a marked and specific local resorption of bone tissue, thus giving morphological evidence of a direct action. Collip, Pugsley, Selye and Thomson (1934) and others (McJunkin *et al.*, 1937) were able to produce bone changes in nephrectomized animals with parathyroid hormone. Moreover, it has been shown by Talmage *et al.* (1953) that nephrectomy appears to nullify the effects of parathyroid extracts upon the serum phosphate level, but the effect on serum calcium is stilll apparent. It is now generally admitted that the hormone may act in both ways (Albright and Reifenstein, 1948). The morphological effects of high doses of parathyroid hormone on bone tissue have been described in detail by *i.a.* Jaffe, Bodansky and Blair (1931), Wilton (1934) and Heller *et al.* (1950), and the effects on the metabolism of calcium and phosphate have been studied by several authors. However, nothing is yet known concerning the mechanism of the action of the parathyroid hormone on bone tissue. In a preliminary communication (Zetterström and Engfeldt, 1951), the present authors have demonstrated a lowering of the renewal rate of bone phosphate in young rats submitted to prolonged parathyroid treatment. In these rats there was also the same

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destruction of old and of young bone as in normal ones. It was the aim of the present investigation to obtain some further information concerning the reaction of bone tissue in experimental hyperparathyroidism using biophysical and chemical methods along with the usual morphological ones.

EXPERIMENTAL

A. *General technique*

In these experiments rats and young dogs were used. The rats were of our own breed, and were kept on a standardized optimal diet with a Ca/P ratio of about 1. Only males were used. In one series of experiments, a study was made of the influence of chronic hyperparathyroidism on the renewal of bone phosphate in rats of different ages. To produce the hyperparathyroidism, they were given 1 U.S.P. unit (Parathyroid extract, Lilly) per gram body weight (weight to the nearest 50 gm.), subcutaneously daily for twenty days. They were separated into four classes according to age, and each class consisted of two treated rats and two controls. 17 hours after the last injection of parathyroid hormone, the rats were given 0.15 or 0.30 millicuries (mc) of radioactive phosphate (P^{32}) as Na_2HPO_4 containing negligible amounts of carrier phosphate. The animals were killed two hours after the injection.

The effect of a single dose of parathyroid hormone on the renewal rate of bone phosphate at different times after the injection was also studied. $2\frac{1}{2}$ months old male rats were given 200 U.S.P. units parathyroid homone, and were killed 6–96 hours after injection. P^{32} was given intraperitoneally two hours before death.

The animals were sacrificed by decapitation. Blood was collected from the carotid arteries, and protein components were immediately removed by precipitation with an equal volume of ice-cold 20% (wt./vol.) trichloracetic acid (TCA). The femurs and tibiae were removed and dissected free of flesh and connective tissue; the diaphyseal and epiphyseal bone were separated and freed from metaphyseal tissue. The bone specimens were frozen in solid carbon dioxide and pulverized in the frozen state. The bone powder obtained was subjected to fractional extraction with saturated ammonium sulphate solution and 15% TCA as described earlier (Zetterström 1952).

For the study of the distribution of mineral salts in bone tissue and the uptake of phosphate in certain structures of bone, dogs were used. Altogether, 6 dogs, 3–6 months old, have been investigated. They received 300 U.S.P. units parathyroid hormone subcutaneously daily for 2–4 weeks, and three hours before death they were injected intravenously with 1 mc P^{32} per kg. body weight. The long bones were taken out, and cross sections were cut from the diaphyses with a rotating saw. The slices were ground on

glass plates to a thickness of about 50μ. Longitudinal sections from the epiphyseal zone were treated in the same way.

B. *Autoradiographic and microradiographic techniques*

Autoradiographic images were produced by placing the sections between two sheets of Agfa Printon film in a special holder which permitted good contact between film and specimen. The film was exposed for about 14 days.

The relative content and distribution of mineral salt was determined by microradiography. The technique was essentially as described by Engström and Wegstedt (1951). A Machlett AEG tube with 1 mm. Be-window was operated at 8 kV to provide radiation with a short wavelength limit of 1.5 Å. The longitudinal sections which were large and rather thick (100–200μ) were microradiographed with a North American Philips diffraction unit with a focus to film distance of 25 cm. The tube was run at 25–40 kV. The microradiograms were registered either on Lippman emulsion or Eastman Kodak spectroscopic plate Nr 649. These emulsions have a resolving power of about 1000 lines per mm. The microradiograms were enlarged by photomicrography using Kodak 0250 plates.

C. *Analytical methods*

The TCA used in precipitating the blood proteins was eventually removed by shaking with ether, and the residual solution was then freed from ether by aeration.

A suitable aliquot of the solution was pipetted into an aluminum dish, dried under an infra-red lamp, and the radioactivity measured using a scaler 64 Electronic Counter yielding 3×10^8 counts per min. per mc P^{32}.

The specific activity (i.e. number of impulses /μg P) of the orthophosphate was derived using the procedure described by Ernster, Zetterström and Lindberg (1950). Phosphate in the bone extracts was determined by the method of Fiske and Subbarow (1925).

RESULTS

Chemical analyses

The effect on the renewal rate of bone phosphate of injecting a single dose of parathyroid hormone was studied at different intervals after injection. In an initial series of experiments, no change in the relative specific activity of bone phosphate of diaphyseal bone could be detected 6 and 12 hours after injecting parathyroid hormone (Table 1). However, as seen from Tables 1 and 2, a definite decrease in renewal rate was demonstrated both in epiphyseal and diaphyseal bone after 18 hours. This decrease in

TABLE 1. RELATIVE SPECIFIC ACTIVITY OF PHOSPHATE IN THE AMMONIUM SULPHATE FRACTION AND IN THE REMAINING FRACTION OF DIAPHYSEAL BONE MINERALS IN TWO CONTROL ANIMALS AND IN THREE ANIMALS AT DIFFERENT INTERVALS AFTER THE INJECTION OF A SINGLE DOSE OF PARATHYROID HORMONE (200 U.S.P.)

Specific activity of orthophosphate in blood = 100. The animals were injected with 0.15 mC P^{32} intraperitoneally two hours before sacrificing. The weight of the animals was 95–100 gm.

Interval after injection of parathyroid hormone	Relative specific activity	
	$(H_4N)_2SO_4$ fraction	Remaining fraction
No injection	17	0.74
No injection	20	0.95
6 hours	20	1.25
12 hours	16	1.13
18 hours	8.8	0.44

relative specific activity persisted for at least 45 hours, but 73 hours after injection the decrease seemed to have diminished, and after 96 hours normal values were again reached.

The reaction of 1, 2, 4 and 8 months old male rats with experimental chronic hyperparathyroidism was studied in a similar way (Table 3). In the two youngest groups, the relative specific activity of epiphyseal and diaphyseal bone phosphate was appreciably diminished, but in 4 months old rats there was no significant change, and the 8 months old rats showed increased relative specific activity in both epiphyseal and diaphyseal bone.

In order to obtain information concerning the incorporation of P^{32} in the bone salt, the relative specific activity of two fractions of the bone salt was determined. The results are shown in Table 4. There were similar changes in the relative specific activity in hyperparathyroidism both in the readily soluble and in the residual fractions. The ratio between the two fractions increased successively with increasing age, indicating that in

TABLE 2. RELATIVE SPECIFIC ACTIVITY OF PHOSPHATE OF EPIPHYSEAL AND DIAPHYSEAL BONE MINERALS IN TWO CONTROL ANIMALS AND IN SIX ANIMALS AT DIFFERENT INTERVALS AFTER THE INJECTION OF A SINGLE DOSE OF PARATHYROID HORMONE (200 U.S.P.)

Specific activity of orthophosphate in blood = 100. The animals were injected with 0.15 mC P^{32} two hours before sacrificing. The weight of the animals was 95–105 gm. The tibial bones were used.

Interval after injection of parathyroid hormone	Relative specific activity	
	Epiphyseal bone	Diaphyseal bone
No injection	7.0	5.7
No injection	5.6	4.6
18 hours	2.2	1.0
22, 5 hours	3.1	1.2
29 hours	1.8	1.2
40 hours	1.7	1.3
45 hours	1.9	1.4
73 hours	4.1	3.5

TABLE 3. RELATIVE SPECIFIC ACTIVITY OF EPIPHYSEAL AND DIAPHYSEAL BONE MINERALS IN NORMAL RATS AND IN RATS OF DIFFERENT AGES WITH EXPERIMENTAL HYPERPARATHYROIDISM

Specific activity of orthophosphate in blood = 100. The animals were injected with 0.15–0.30 mC P^{32} two hours before sacrificing. The treated animals had been given daily injections of parathyroid hormone for twenty days. Tibiae and femurs were used.

Age in months when treatment is started	Relative specific activity			
	Epiphyseal bone		Diaphyseal bone	
	Controls	Treated	Controls	Treated
1	18	6.8	6.5	2.6
2	9.4	5.6	5.1	3.4
4	4.4	4.0[a]	2.6	2.5[a]
8	1.1	3.3	0.6	1.6

Each value represents two animals.
[a] Only one animal.

older animals a smaller amount of the given P^{32} is incorporated in the bone salt. The results with the parathyroid treated animals were inconclusive, but there were indications of a decreased incorporation in the youngest group and an increase in the oldest.

Microradiography and autoradiography

Microradiograms from the cross sections of bone tissue from the diaphyses of normal young dogs gave clear information on the distribution of minerals salts in different structures. Many osteons showed differences in mineralization as indicated by different absorption of the x-rays used. The young newly-formed osteons had less absorption, and were thus shown to be less mineralized than older osteons. For further information concern-

TABLE 4. RELATIVE SPECIFIC ACTIVITY OF PHOSPHATE IN THE AMMONIUM SULPHATE FRACTION AND IN THE RESIDUAL FRACTION OF EPIPHYSEAL AND DIAPHYSEAL BONE MINERALS IN NORMAL RATS OF DIFFERENT AGES, AND IN COMPARABLE RATS WITH EXPERIMENTAL HYPERPARATHYROIDISM

Specific activity of orthophosphate in blood = 100. The experimental conditions are the same as in Table 3.

Age in months when treatment was started	Relative specific activity							
	Epiphyseal bone				Diaphyseal bone			
	$(H_4N)_2SO_4$ fraction		Remaining fraction		$(H_4N)_2SO_4$ fraction		Remaining fraction	
	Controls	Treated	Controls	Treated	Controls	Treated	Controls	Treated
1	51	20	5.1	1.5	27	11	2.5	0.84
2	40	19	2.6	1.6	24	23	1.9	0.83
4	26	18[a]	1.3	1.2[a]	13	20[a]	0.82	0.88[a]
8	8.4	22	0.30	1.1	3.8	7.4	0.18	0.34

Each value is the mean of the determinations from two minerals.
[a] This value is only one determination.

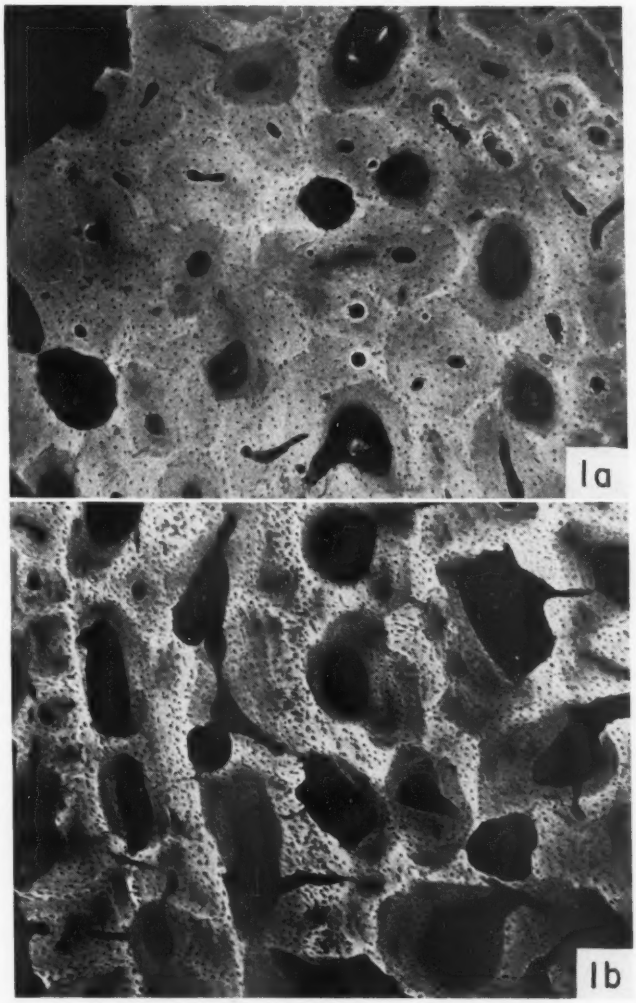

FIG. 1. Microradiograms of 50μ thick cross sections of the diaphyses from tibiae of dogs. (a) Normal dog. Only a few resorption cavities can be seen. 33×. (b) Dog treated with parathyroid hormone. Many resorption cavities and also widespread areas with a low mineralization. 33×.

ing the normal microradiographic picture see Amprino and Engström (1950).

The microradiograms from the parathyroid treated dogs showed a bone tissue which was much more irregular than normal bone tissue from dogs

of the same age. There were many large resorption cavities with somewhat irregular boundaries (Fig. 1 a and b). One could also frequently demonstrate Haversian systems with low mineralization. In such systems a rebuilding of bone tissue was indicated which was at a level as pronounced in treated animals as in the normal ones.

The autoradiographic study of cross sections of bone tissue from long bones of dogs revealed small but significant differences between the normal animals and those with chronic hyperparathyroidism (Fig. 2a and b). The autoradiographic images of normal bone obtained with P^{32} showed a high uptake of isotope in the growing parts of the bone. In compact bone tissue there was also a localized uptake in areas corresponding to young Haver-

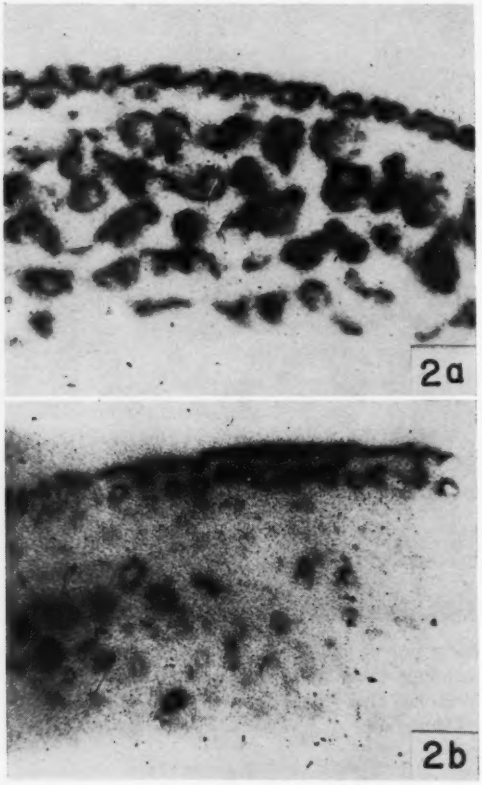

FIG. 2. Autoradiograms of cross sections of the diaphyses from tibiae of young dogs. (a) Normal dog. The periosteum and most of the Haversian systems show a high uptake of isotope. 8×. (b) Dog treated with parathyroid hormone. Only few Haversian systems show appreciable uptake of isotope. 8×.

sian systems i.e. in systems with low content of mineral salts (Engfeldt, Engström and Zetterström, 1952). In the bone from hyperparathyroid dogs, periosteal and endosteal regions showed a broad zone of blackening, indicating uptake of radiophosphate in these regions, but in the compact bone only a few osteons showed a detectable uptake. This uptake was localized in a zone around the Haversian canals. Very often the blackening corresponding to the Haversian system was only evident in parts of its circumference.

DISCUSSION

The difference in the renewal rate of bone phosphate in young parathyroid treated rats in comparision with normal controls, and the observations made from the autoradiographic study of the bones of dogs treated with parathyroid hormone for long period, are in good agreement. The incorporation of P^{32} in the bone salt of young parathyroid treated animals is decreased, thus indicating a diminished renewal rate of bone phosphate.

The reactions to parathyroid hormone of different age groups of rats showed definite differences between young and old animals. The young animals reacted to chronic hyperparathyroidism with a decrease in renewal rate of bone phosphate, whereas the old rats showed an increased renewal. These findings may be related to the differences in rate of growth in the different age groups. As shown by the microradiograms in the case of dogs, chronic hyperparathyroidism produces increased bone destruction, but there are also features indicating building of new bone tissue.

A general explanation of these observations can be made by assuming that the primary action of parathyroid hormone is an acceleration of bone destruction covering both old and newly formed bone tissue. And further on that an overall decrease in the amount of bone tissue eventually leads to an increased rate of bone formation. Thus, in young animals, with their rapid bone formation, the increased destruction will cover a large amount of newly formed tissue, the net effect being an apparent decrease in the rate of production of new bone. In old animals, however, when the bone metabolism is relatively static, the increased rate of destruction produced by the parathyroid hormone soon leads to a decrease in the total amount of bone in the system, and this in turn causes an increase in the rate of bone formation. The net effect is thus an increase in the rate of renewal of bone. These results are in agreement with the hypothesis put forward by Albright and Reifenstein (1948). The differences in reaction of different age groups to parathyroid hormone must be kept in mind when interpreting experimental results. It is possible that the inability of Tweedy and Campbell (1944) to find any difference in renewal of bone phosphate in normal and hyperparathyroid rats resulted from this fact.

From the microradiograms, it is clear that the destruction of bone tissue resulting from the effects of the parathyroid hormone affects both young newly formed osteons and old ones. This is also true in normal bone destruction where resorption cavities often occur which cover several osteons with different degrees of mineralization. It has also been demonstrated that the ratio between resorption of newly formed bone tissue and old bone is of the same degree in hyperparathyroidism as in normal animals. Thus, the characteristics of bone destruction in hyperparathyroidism are essentially the same as in normal bone, and the observed effects may result simply from an acceleration of the normal process of bone destruction.

The present results indicate clearly the processes ultimately affected by the parathyroid hormone, but the detailed mechanism of its action on bone tissue remains obscure. X-ray diffraction studies have so far failed to reveal any changes in the bone ultrastructure (Engström and Engfeldt, 1951). On the other hand there are several pieces of indirect evidence in favour of a primary action of the hormone on an organic constituent of bone tissue. Morphological changes have been observed after six hours, whilst the changes in relative specific activity of the bone phosphate are not appreciable until eighteen hours after the injection of a single dose of parathyroid hormone, thus indicating a primary action on cell constituent. The fact that the bone destruction in hyperparathyroidism does not seem to differ in type but only in extent from the normal could also be taken as evidence in favour of a cellular action of the parathyroid hormone.

SUMMARY

The effects of parathyroid hormone on bone tissue have been investigated by studying the rate of renewal of bone phosphate using chemical and autoradiographic techniques, and the general distribution of mineral salts using microradiography. In young animals the rate of renewal of bone phosphate is decreased but is increased in the case of old animals. The destruction of bone tissue is accelerated by the parathyroid hormone, but the characteristics of the process are unchanged. The interpretation and implications of the results are discussed.

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ELECTRON MICROSCOPIC STUDIES OF THE ANTERIOR PITUITARY GLAND OF CASTRATE RATS[1]

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INTRODUCTION

MANY features of the cytology of the anterior pituitary gland, its reactions to physiological stimulation and removal of other endocrines, remain obscure in spite of the extensive research that has been carried out on the subject during the past seventy years.

Progress in such a field of study often awaits the advent of new techniques. With a greater understanding of the chemistry of pituitary hormones it has become possible, at least in a limited way, to apply histochemical methods to aid in identification of the cell source of a given hormone. Quite recently, the application of electron microscopy to histologic and cytologic investigation has opened a new field for study. The remarkable structural detail that can be seen in electron micrographs offers much promise in histologic research.

Fernández-Morán and Luft (1949) recorded the first electron microscopic observations on the pituitary gland. However, at that time adequate sectioning techniques had not been developed, and their observations were restricted to material prepared by smear and replica techniques of limited usefulness.

Recently a preliminary report was made on the cytology of the anterior pituitary gland of normal rats as revealed by the electron microscopic examination of ultrathin sections (Rinehart and Farquhar, 1953). All of the known cellular elements—nuclei, nucleoli, mitochondria, granules, vesicles, cytoplasmic filaments—may be seen in exquisite detail in a single preparation and often in a single field. Thus, the ability to differentiate cells has been greatly enhanced. What is even more important, the use of the electron microscope offers promise in clarifying obscure problems in cytophysiology.

Of all the observations made to date on experimentally produced cytologic alterations in the pituitary, those most in agreement pertain to the pituitaries of gonadectomized rats. For this reason the study of cellular

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changes in the hypophysis of the castrate rat was chosen as the first in a series of experimental studies of altered pituitary cytology. Thus, we hoped to add to the information already learned from light microscopy and, by so doing, to aid in the interpretation of cytological features noted in our previous electron microscope studies on the normal rat pituitary.

The changes in the pituitary of the rat following castration have been described in detail by numerous investigators (see Severinghaus, 1940, for a review of the literature). Although differences of opinion existed among these workers regarding details and interpretations of their findings, all of them described an increase in the size and number of the basophils in the castrate animal.

More recently, histochemical staining techniques have been applied to the anterior pituitary of the castrate animal. Catchpole (1949) applied to the rat hypophysis the McManus periodic acid-Schiff's reagent (PAS) for demonstration of glycoproteins, and his studies were followed by those of Purves and Griesbach (1951a) on the same animal. Halmi (1950) and Purves and Griesbach (1951b) similarly applied Gomori's paraldehyde-fuchsin staining method. All of these studies utilizing histochemical techniques point to the existence of additional cell types in the anterior pituitary of the rat. Specifically, Purves and Griesbach describes two types of cells ordinarily identified as "basophils" by classical staining methods: a "gonadotroph" (the same cell Halmi (1952) called a "delta" cell) which responds specifically to physiological variations in gonad activity, and a "thyrotroph" (called a "beta" cell by Halmi)which responds to physiological alterations in thyroid activity.

The purpose of this paper is to illustrate the cytologic changes produced in the pituitaries of castrate rats as revealed by the electron microscope and to discuss the physiological significance of these findings.

MATERIALS AND METHODS

Sixty adult female rats of the Sprague-Dawley strain were castrated at two months of age and sacrificed at intervals of 6, 10, 20, 35 and 75 days following castration. The animals were anesthetized with ether and sacrificed by exsanguination. The pituitaries were removed immediately and immersed in a solution of 1% osmium tetroxide (osmic acid) buffered at pH 7.4 with acetate-veronal buffer, according to the method of Palade (1952a). While the tissue was immersed in this solution, segments of about 1 cu. mm. were cut, and the pieces obtained from each gland were placed in individual vials containing 3–5 cc. freshly prepared, buffered osmic acid solution.

The tissue was fixed for four hours, washed in several changes of distilled water for one hour and dehydrated in several changes of 70, 95 and 100% alcohol for periods of one hour each. From absolute alcohol the tissue was placed in a mixture of equal parts absolute alcohol and the monomer n-butyl methacrylate (containing 1% of the catalyst 1,2, dichlorobenzoyl peroxide) for one hour and then into three changes, one hour each, of n-butyl methacrylate. The tissue was then placed in 00 gelatin capsules partly filled

with n-butyl methacrylate or n-butyl methacylate containing 5% methyl methacrylate (to increase hardness of the block) and put in an oven at 47°C. overnight to polymerize.

The polymerized block was then allowed to harden at room temperature for a period of one to two weeks before it was trimmed and sectioned with a Spencer Rotary Microtome, according to the thermal expansion method of Newman, Borysko and Swerdlow (1949), using a ⅜-inch plate glass knife (Latta and Hartmann, 1950). The microtome was recently equipped with an improved knife holder similar to that devised by Dempsey and Lansing (1953). Sections were cut directly into a bath containing acetone and water or water alone. The thinnest sections were selected, and some were picked up onto collodion-coated screens (200 mesh/inch) and examined in the electron microscope without removal of the embedding medium. Other sections were placed on a drop of water on a slide, allowed to dry and then dipped into .2% collodion in amyl acetate to remove the methacrylate and substitute collodion. The membrane and tissue were floated onto water; screens were placed on the tissue sections, and the screens containing the tissue were put on filter paper to dry.

When the sections were dry they were examined with an RCA EMU-2B Electron Microscope equipped with a wide angle pole piece for increased contrast (Hillier, 1951), photographed at magnifications ranging from 300× to 5000× and, thereafter, enlarged photographically as desired up to 25,000×.

Several pituitaries from the 10-, 20- and 35-day castrates and normal controls for each group were fixed for 24 hours in formol-sublimate, embedded in paraffin, sectioned at 3–4μ and examined by light microscopy after staining with either hematoxylin and eosin, periodic acid-Schiff reagent (Hotchkiss, 1948) or an acetaldehyde-fuchsin technique (Abul-Haj and Rinehart, 1952).

In addition, 1μ sections were cut from the osmic acid-fixed, methacrylate-embedded material. Some of these sections were dipped in acetone to remove the methacrylate, hydrated, stained for five minutes in Ehrlich's hematoxylin, dehydrated, cleared and mounted for light microscopy. Other 1μ sections were just cleared and mounted for study with the phase microscope. These sections aided in orientation of sections from the same block seen in the electron microscope. Many details of cellular structure visible under the electron microscope could be seen in the osmium-fixed 1μ sections examined in the phase microscope that could not be seen in material prepared for the light microscope. The greater detail apparent in the sections prepared for phase microscopy was attributed to their relative thinness and the excellent state of preservation afforded by the osmium fixation.

OBSERVATIONS

Before describing changes produced in the rat anterior pituitary by castration, it is first necessary to describe a few important cytologic features seen in the normal female rat pituitary with the electron microscope. More detailed observations have been recorded elsewhere (Rinehart and Farquhar, 1953).

Three classically described cell types of the anterior pituitary are the acidophils, basophils and chromophobes. With the electron microscope the acidophils and basophils are readily identified by the difference in size of the granules they contain. The acidophil granules are the coarsest and are of a relatively uniform size and density, having a maximal diameter of

approximately 350 mμ. The basophil granules are much finer, their maximal diameters varying from 140 to 200 mμ in different cells. In light microscopy, cells without visible granules are classed as chromophobes. With the election microscope very few true chromophobes are seen, for most cells classed as such with the light microscope when viewed with the electron microscope are seen to contain variable numbers of granules of a characteristic size which would place them in either the acidophil or basophil series (Fig. 14). This observation concurs with that of Severinghaus (1933), who identified chromophobes as belonging to either the acidophil or basophil series by the type of Golgi apparatus they contained. Based on his observations, Severinghaus postulated a secretory cycle for the acidophil and basophil cells as two independent cellular elements, the sequence being granule accumulation and storage and their subsequent secretion after an effective stimulus, thereby returning the cell to the chromophobic or nongranular state.

In addition to the well known cytoplasmic organelles—the Golgi apparatus and mitochondria—fine double-membraned canalicular structures (ergastoplasmic filaments) are sharply delineated in certain cells of the anterior pituitary. Such structures are illustrated in this and in our preceding report. Further consideration of this most interesting cytoplasmic structure is set forth in the discussion.

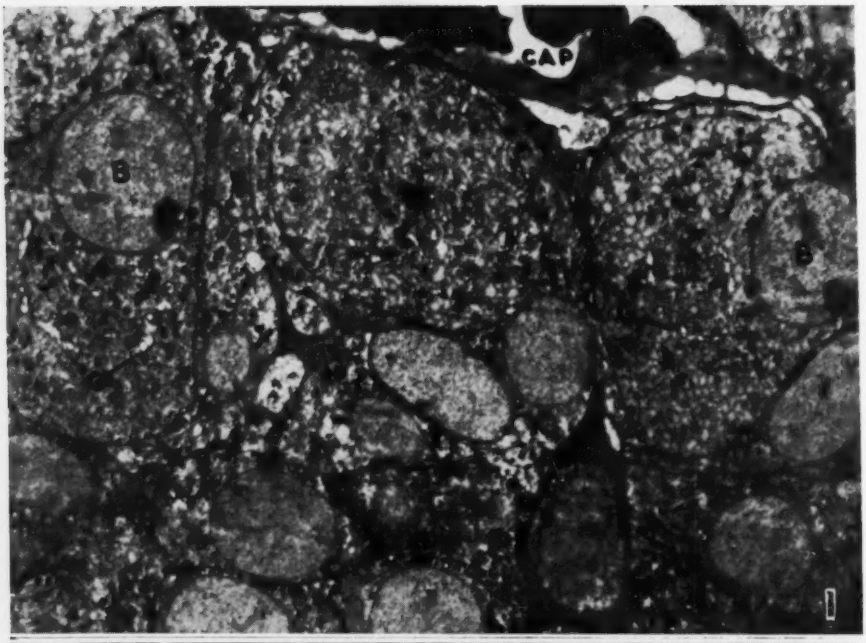
BASOPHILS

Since the appearance of the castration basophils is fairly characteristic for each interval after castration, the observations on the castration cells will be described under time interval headings.

Six-day Castrates

As early as six days after castration a low power examination of any field with the electron microscope reveals the presence of increased numbers of enlarged vesiculated cells containing fine granules (Fig. 1). Although a few such cells are seen in different stages of granule accumulation and depletion, the majority of these cells appear to be in practically the same phase, as evidenced by their similar size, amount of granular and vesicular material, nuclear density and appearance of the Golgi apparatus.

The "typical" six-day cells of castration are larger than any cell yet seen in the normal female rat pituitary, often achieving a maximal diameter of 15–20μ. They have rounded contours and usually are seen to lie adjacent to a capillary (Figs. 1, 2). The nuclei are rounded, of a light but even density, lie closer to one end of the cell and usually contain one or sometimes two prominent nucleoli with irregular margins and a "knobby" texture. The nucleolus lies close to the border of the nuclear membrane.


CAP
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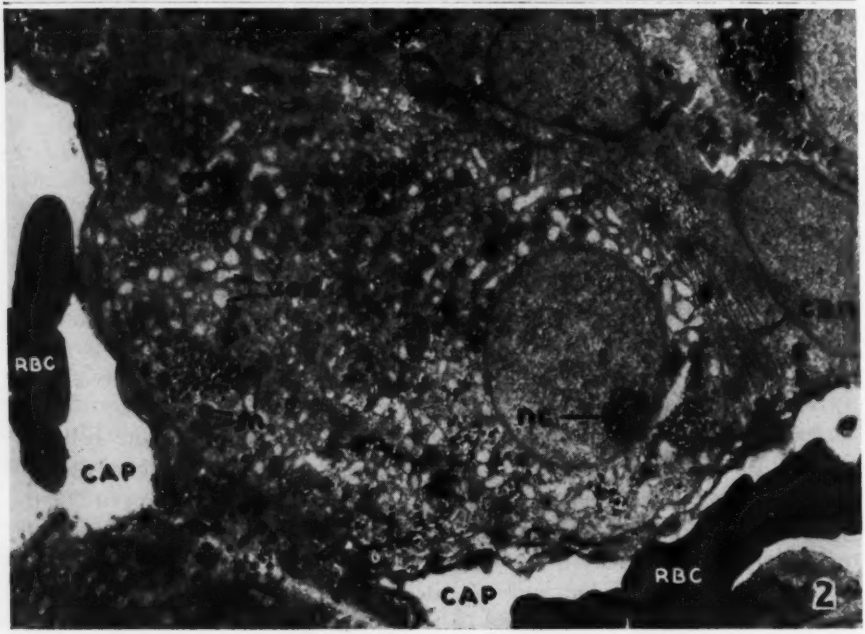
1


RBC
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2

Since these criteria have served to distinguish gonadotrophic basophils or "gonadotrophs" (Halmi, 1950; Purves and Griesbach, 1951a), they will be so designated in subsequent discussion.

In the cytoplasm of the gonadotrophs, variable numbers of tiny vesicles are found embedded in an amorphous ground substance. The cytoplasm of many of these cells consists almost entirely of tiny vesicles between which lie clumps of fine granules and numerous mitochondrial rods and filaments, seen sectioned in various planes (Fig. 2). The mitochondria are found throughout the cytoplasm but often are concentrated in and around the region of the Golgi apparatus (Figs. 4, 5, 6).

At this stage of the Golgi apparatus is found near the nucleus and is outlined by a circular chain of tiny vacuoles which appear to be empty or have no inherent density when compared with the cytoplasmic vesicles, which exhibit a light through definite density (Fig. 1).

Ten-day Castrates

The enlarged gonadotrophs of the ten-day castrate appear quite similar to those of the six-day castrate. Most of the vesicles are approximately the same size as those seen previously, but some appear slightly larger (Fig. 3). There is additional complication and enlargement of the Golgi apparatus. Sometimes there is also an increase in the density of the cytoplasmic ground substance in the Golgi area.

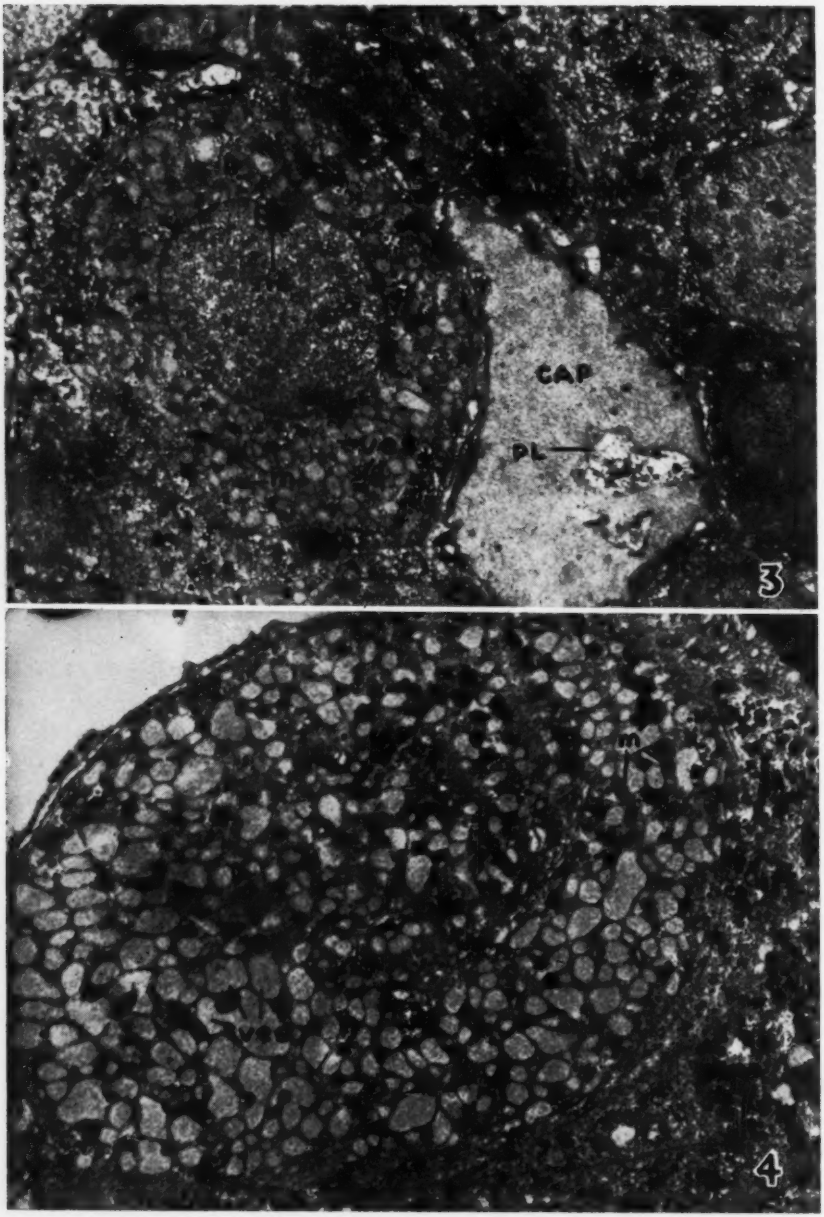
The size and shape of the cells, their location, the nuclear and nucleolar position and size and the distribution of the mitochondria do not appear to differ significantly from those seen in the pituitaries of six-day castrates.

←

FIG. 1. Six-day castrate. This lower power figure shows three enlarged gonadotrophic basophils (B). These cells are seen to have rounded contours, and two of them lie on the capillary (CAP) above. Two of the cells contain eccentrically placed, light nuclei with prominent nucleoli. The cytoplasm of all three of these cells is seen to be almost entirely filled with fine vesicles of a light density between which lie variable numbers of granules. Numerous mitochondria are distributed throughout the cytoplasm. The Golgi apparatus (G) in each cell is outlined by a circular, beadlike, vacuolar chain. ×3500.

FIG. 2. Six-day castrate. A greatly enlarged gonadotroph is shown in higher magnification. The cell has a rounded contour and lies on a sinusoid or capillary (CAP) which contains several red blood cells (RBC). It shows an eccentrically placed nucleus and a prominent nucleolus (nc) of an irregular or "knobby" texture. The nucleolus is seen to lie close to one border of the nuclear membrane. In the cytoplasm numerous fine granules (gr) and mitochondria (m) are seen between many tiny vesicles (ves.) This cell is of particular interest in that it appears to show transitions between canalicular structures and vesicles.

To the right of the gonadotroph is an acidophil in which very delicate canalicular structures (can) are seen arranged in parallel ×6000.


CAP
PL
3
M
4

Twenty-day Castrates

In the twenty-day castrate pituitary more variation is encountered in the appearance of the hypertrophied gonadotrophs. Some resemble those described as typical of the pituitaries of six- and ten-day castrates (Fig. 5). Many show a more pronounced degree of vesiculation of the cytoplasm (Fig. 4). The vesicles are now larger and have less rounded borders, appearing as if adjacent vesicles had coalesced to form larger and more irregular structures (Figs. 4 and 5). In addition, the size and internal density of these bodies vary considerably from cell to cell, although they are fairly constant within any one cell. The granule content seems to diminish in quantity as the average size of the vesicles increases. The granules that remain are distributed throughout the cytoplasm but are more concentrated in the area within and around the Golgi apparatus and at the periphery of the cell. The mitochondria retain their previous form, density and distribution.

The Golgi apparatus stands out more prominently in the gonadotrophs of the twenty-day castrate (Fig. 5). This is due in part to a decrease in the density of the cytoplasmic elements brought about by increased vesiculation with a loss of granular content and, in part, due to a complication and enlargement of the structure of the Golgi apparatus itself, Previously, the Golgi apparatus was smaller and outlined by a circular beadlike chain of vacuoles; now it is crossed by strands of the vacuolar chain which forms S-shaped configurations inside the circle (Fig. 5). The concentration of granules and mitochondria and the increased density of the ground substance in the area give it the appearance of being a center of cellular activity.

The structure of the nucleus remains much the same, except that the nuclear membrane of some cells becomes serrated in appearance.

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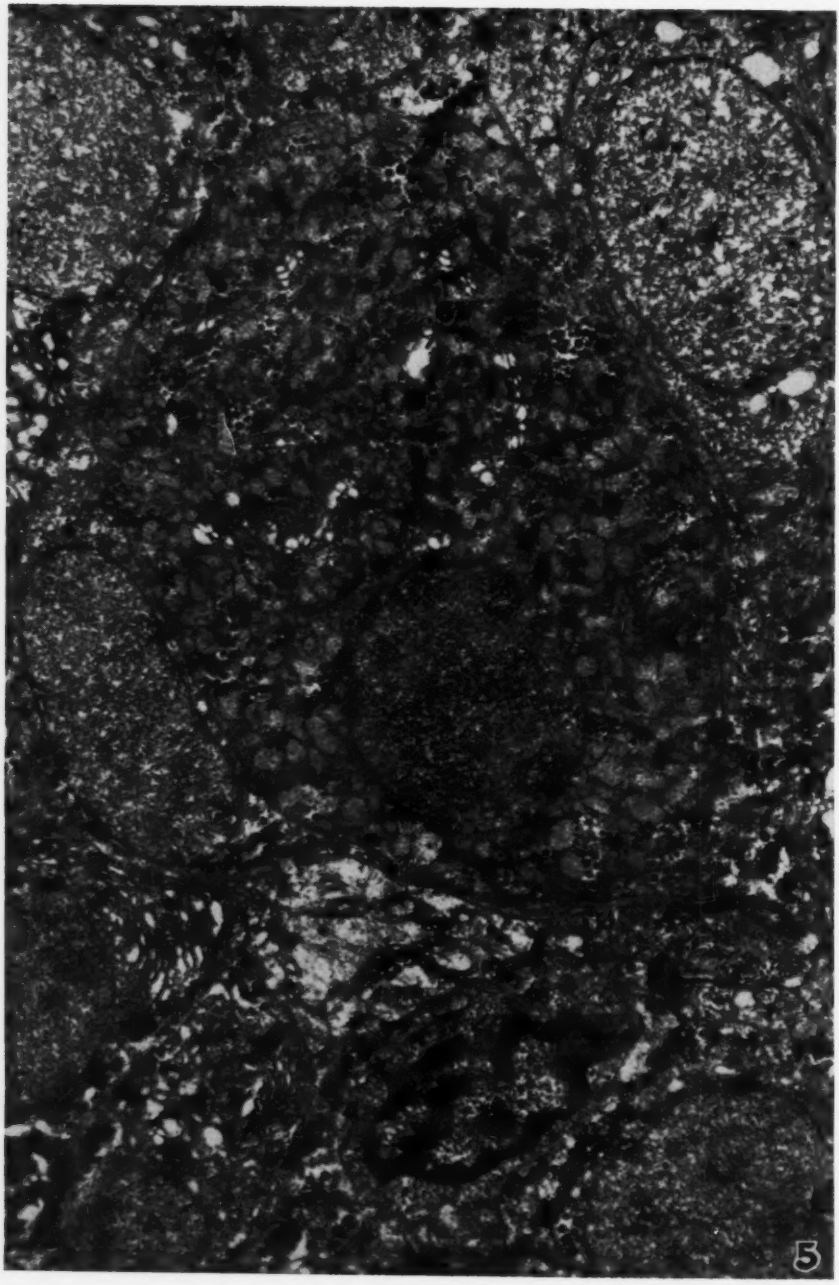
FIG. 3. Ten-day castrate. It is seen that this gonadotroph is very similar to the cells prominent in the pituitaries of the six day castrate (Figs. 1 and 2). This cell is located on a capillary (CAP) and contains cytoplasmic vesicles (ves) with numerous mitochondria (m) and some granules. The nucleus contains a dense nucleolus (nc) and shows a prominent nuclear membrane.

The capillary (CAP) contains portions of several blood platelets (PL). The platelets have a well defined membrane and a light cytoplasm with irregular granular densities. ×5000.

FIG. 4. Twenty-day castrate. Larger vesicles (ves) fill the cytoplasm of this gonadotroph. These larger vesicles appear to have arisen by a coalescence and pooling of smaller and rounded units.

A large Golgi area is included. Numerous mitochondria (m) are concentrated in and around this area. The granule content is seen to be diminished in the presence of larger vesicles. The granules which remain are found chiefly in the Golgi area or along the cell membrane. ×5000.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Thirty-five- and Seventy-five-day Castrates

The anterior pituitaries of these later castrates are described together since the pituitary of the seventy-five-day castrate differs from the thirty-five-day castrate only in the presence of increased numbers of cells in more advanced stages of vesiculation.

In the pituitaries of both of these groups of animals are found a few of all of the cells described as being prominent in the six-, ten- and twenty-day castrates. However, many of the gonadotrophs have progressed to a more grossly vesiculated state where increased numbers of single units have coalesced to form larger bodies (Figs. 6 and 8). This process of condensation of the finer vesicular elements ultimately results in some of the cells becoming almost entirely occupied by one or several large vesicles giving the typical "signet-ring" appearance often described in light microscopy.

Electron microscopy reveals considerable differences in cells which in light microscopy are known as signet-ring cells. Some of the cells consist almost entirely of multiple, rather large vesicles of light density which fill the cytoplasm of the cells so that the only other elements remaining are a few short rodlike mitochondria and granules concentrated in the Golgi are (Fig. 6). These larger vesicles are separated only by a few delicate strands of cytoplasm. Other cells with one or more large vacuoles have larger quantities of cytoplasm containing many mitochondria, granules and smaller vesicles. Cells of this description look more like a signet ring in electron micrographs (Fig. 7). The material in the large spaces varies in density from cell to cell and occasionally within the same cell. In general, however, the density of this material seems to diminish as the average size of the spaces increases within the cell.

In the pituitaries of the later castrates other vesiculated cells are found which are quite different in appearance from those previously described.

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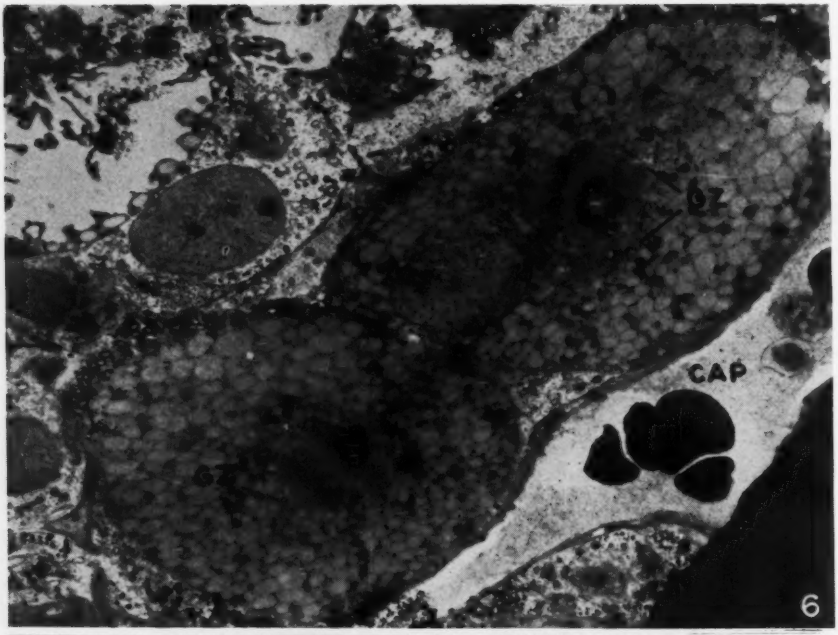
FIG. 5. Twenty-day castrate. A hypertrophied gonadotroph occupies most of this field. This cell does not differ materially from those seen in the ten day castrate, with the exception that a few of the vesicles in the cytoplasm below the nucleus appear to be enlarging by coalescence of smaller units. In addition, the Golgi apparatus is greatly enlarged and shows coarser vacuoles (GV). Its circular vacuolar chain is crossed by other vacuoles giving it an S-shaped configuration. Numerous granules (gr) are present. Mitochondrial rods and filaments (m) are seen throughout the cytoplasm but are found particularly in and around the Golgi apparatus.

Below the gonadotroph and to the left are portions of two acidophils (A) showing coarsened canaliculi.

Just below the gonadotroph and to the right can be seen a very small portion of the cytoplasm of another "basophil" which contains granules of a significantly smaller size than the granules of the adjacent large castration cell. ×6500.



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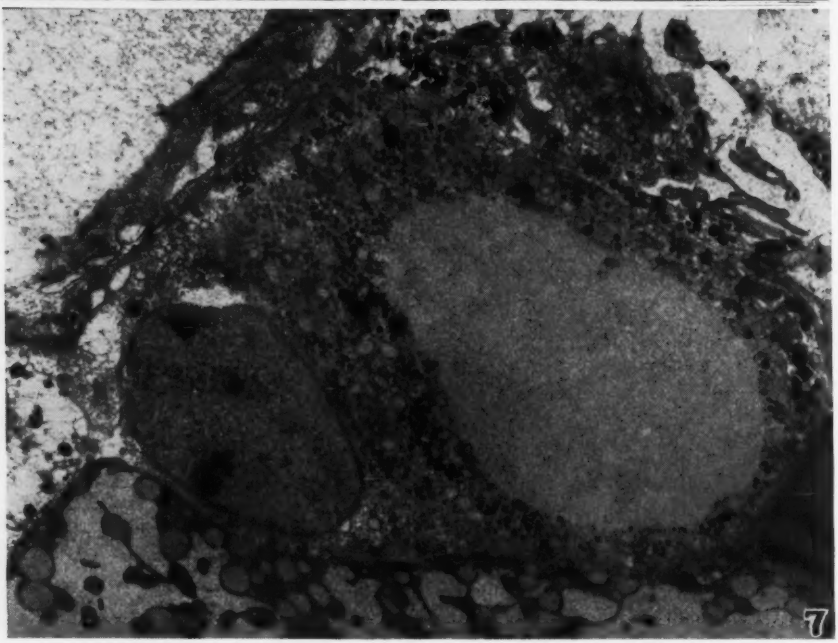

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This second type of cell is characterized by dense, elongated or folded nuclei and bizarre cytoplasmic formations resembling exquisitely delicate filigree (Figs. 9–12). The nucleus is usually quite dense, so that the nucleolus may barely be visible against the dark nuclear background. The cytoplasm consists of strands or filaments along which are found irregular clumps of small granules and vesicles. Small munbers of such cells have been seen in glands of normal rats. These cells are thought to be "gonadotrophs" since they possess the morphologic criteria ascribed to these cells —location on vessels, rounded contours, etc. Another feature which distinguishes these filigreed cells as belonging to the gonadotrophic group is the observation that they undergo characteristic alterations after castration. Following castration these cells also enlarge and show varying degrees of vesiculation of the cytoplasm coupled with granule loss. In general, however, the granule content of such cells is greater at any particular stage of vesiculation than the granule content in the previously described gonadotrophs. In addition, the vesicles instead of being rounded are typically quite irregular in shape. As the granule content of such cells decreases, the vesicular spaces increase in size until ultimately a cell remains which, in light microscopy, would be called a signet-ring cell. On examination with the electron microscope, however, it is seen that they are quite different in appearance from the signet-ring cells previously described (compare Fig. 7 with Figs. 11 and 12.)

The nuclear and cytoplasmic variations characteristic of the filigreed cells are also identifiable in stained 1 μ sections examined with the phase microscope.

ACIDOPHILS

From an examination of numerous low power fields, we have noted that there are lesser numbers of heavily granulated acidophils present in the rat pituitary following castration. Up to 35 days after castration a progres-

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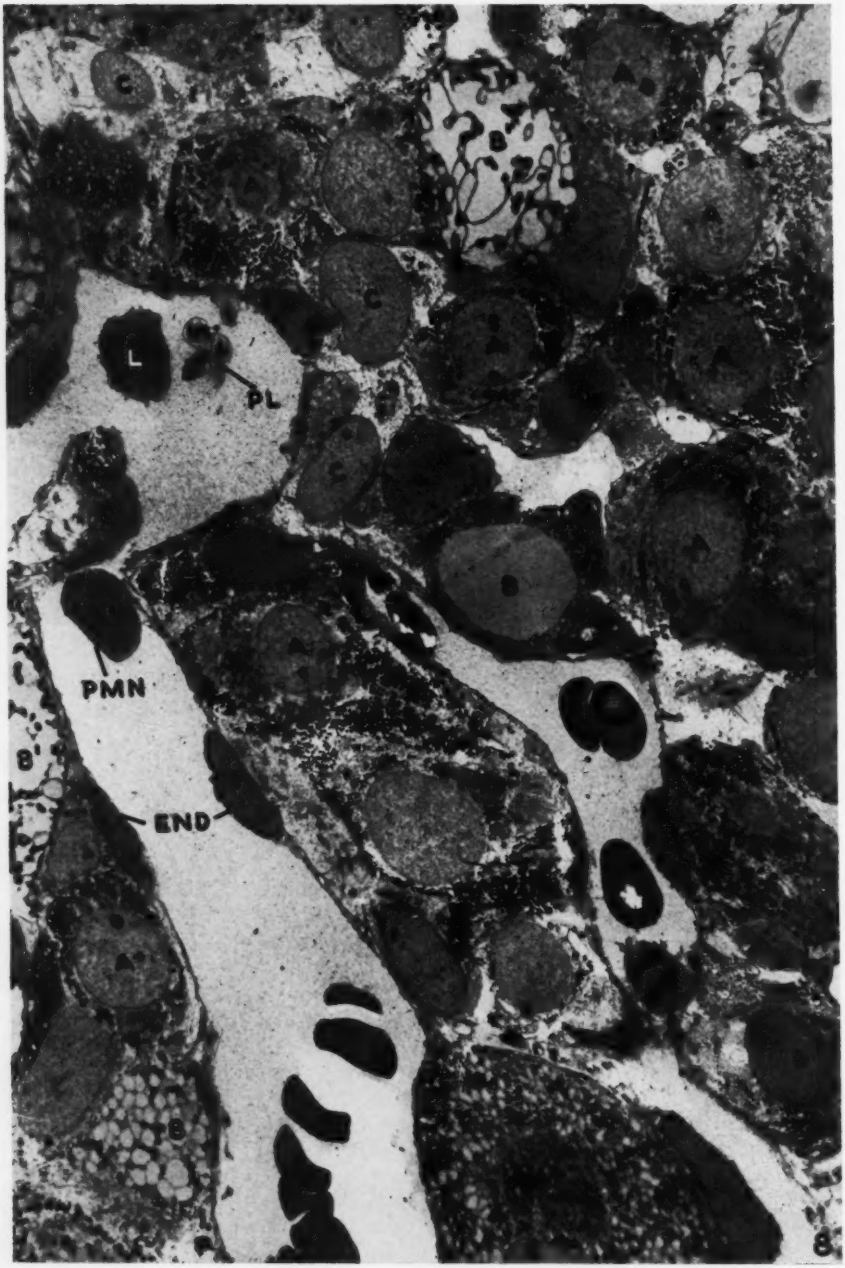
FIG. 6. Seventy-five-day castrate. Two vesiculated gonadotrophs are shown bordering a capillary (CAP). The cytoplasm of these two cells is filled with rounded vesicles. A few granules are seen, chiefly in the Golgi zones (GZ) and along the cell membranes. The Golgi zone of both cells stands out clearly against the lighter cytoplasmic background. Numerous mitochondria which are mostly short rods are found throughout the cytoplasm but are particularly prominent in the Golgi area. A nucleus of light homogeneous density is visible in the cell on the right. ×3500.

FIG. 7. Seventy-five-day castrate. This figure illustrates a gonadotroph which has a "signet-ring" appearance. The cytoplasm contains a single large vesicle surrounded by a rim of cytoplasm which contains numerous granules, mitochondria and smaller vesicles.

Below is a portion of a gonadotroph (B′) showing a bizarre cytoplasmic structure resembling filigree. ×6000.


L
PL
PMN
END

sive increase in the numbers of partially degranulated acidophils is seen, and fewer numbers of heavily granulated cells are evident. Acidophils with a single row of granules lined up along the cell border are numerous (Figs. 14 and 17). These cells usually do not contain a Golgi apparatus.

The degranulated acidophils show altered mitochondria. They are large, spherical and of relatively light density. Mitochondrial filaments have not been seen in these cells, and rods are seldom seen. These degranulated acidophils often contain prominent canalicular structures (ergastoplasmic filaments) (Fig. 16). The circular arrangement of cytoplasmic filaments around a central mass or core constitutes the "Nebenkern" (Fig. 15). At 75 days after castration there is an apparent "recovery" with increased numbers of heavily granulated acidophils becoming evident (Fig. 8).

In addition to the type of acidophil seen in the control animals, other "acidophils" were identified in the pituitaries of castrates. The latter contain granules of a significantly larger size than those which we described previously, and the granules are elliptical rather than rounded in shape (Fig. 13). These larger particles measured up to approximately 600 mμ in their longest dimension as contrasted to a maximal diameter of 350 mμ found for the previously described acidophil granules. These large elliptical bodies are also often found in cells with a prominent or hypertrophied

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FIG. 8. Seventy-five-day castrate. This low power figure illustrates a number of acidophils and basophils in varying secretory phases.

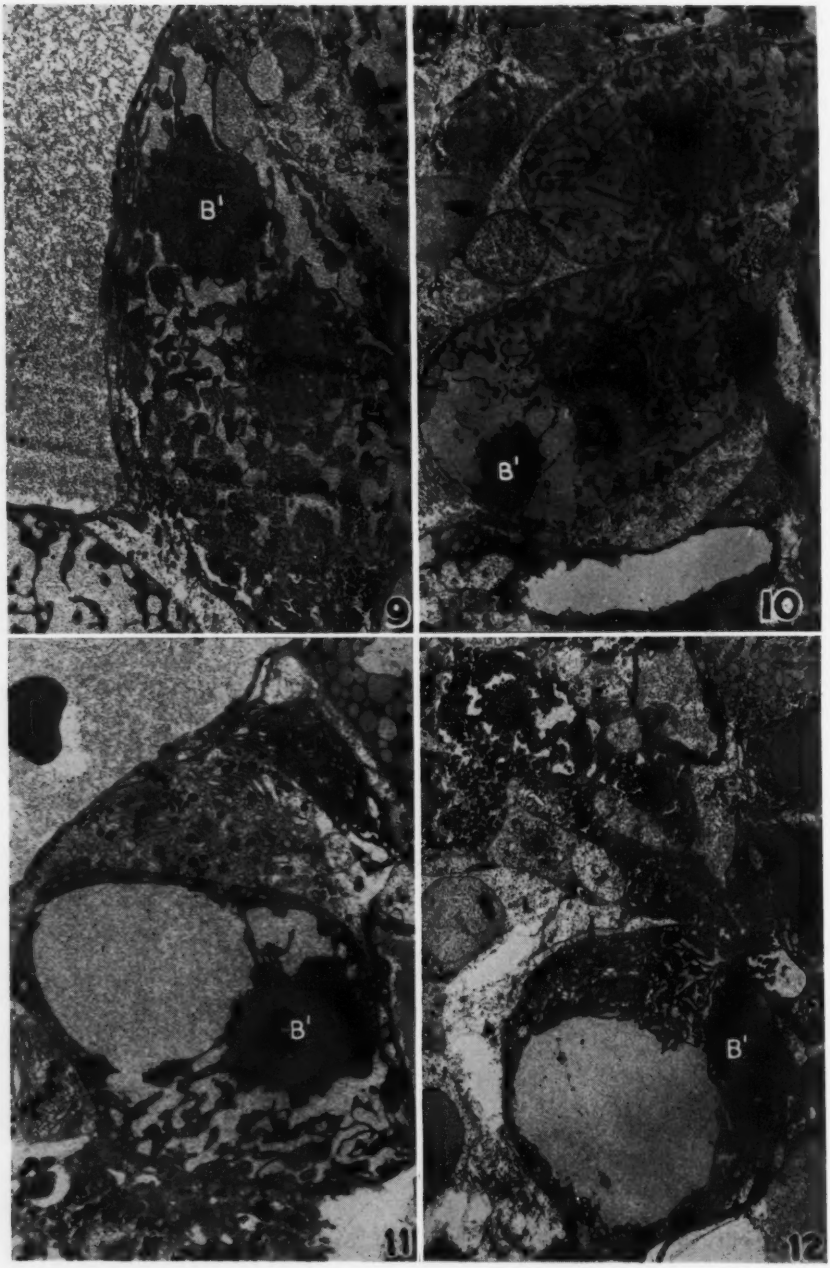
Numerous acidophils (A) with their large, dense, osmiophilic granules are seen scattered throughout the field. Some are packed with granules (upper left, upper right, lower left), and others show a lesser number of granules (upper right, center right).

A number of gonadotrophs (B) are shown. In the center of the field is a "signet-ring" castration cell consisting of a large vesicle surrounded by a small rim of granular and vesicular cytoplasm. The nucleus of this cell is not visible. At the bottom of the field in the center and adjoining the capillary is part of another large gonadotroph with vesiculated cytoplasm. Considerable numbers of granules and mitochondria are seen in this cell. A prominent Golgi zone and part of the nucleus are also visible. In the lower left corner to the left of the capillary is a portion of the cytoplasm of another vesiculated cell. These three gonadotrophs are thought to be F.S.H. producers.

In the center at the top of the figure is part of the cytoplasm of another gonadotroph (B′). It presents a bizarre lacelike cytoplasmic structure resembling filigree and contains a few granules and vesicles. This cell is thought to represent an L. H. producer.

Several chromophobic or sparsely granular cells (C) are seen in the field in the upper left corner and to the left of the signet-ring cell.

Two capillaries are prominent in this field. The elongated homogeneous nuclei of several endothelial cells (END) are seen along the capillary walls. Numerous dense red blood cells are visible in the lumina. In the upper part of the large capillary on the left a lymphocyte (L) and a cluster of blood platelets (PL) are seen. Below is a polymorphonuclear neutrophil (PMN) containing small dense granules and a coiled nucleus. ×2500.


B'
9
B'
10
B'
11
B'
12

canalicular apparatus and, rather often, are seen as the central core of a "Nebenkern" (Fig. 13).

DISCUSSION

Observations by light microscopy have shown that after castration there is an increase in the size and number of the gonadotrophic cells in the anterior pituitary of the rat. Observations by electron microscopy indicate that after castration the stimulated cells undergo a regular sequence of structural changes characterized by a decreasing granule content with increasing vesiculation of the cytoplasm.

Ellison and Wolfe (1934) and Reese, Koneff and Wainman (1943) observed the early vesiculation of the basophil cytoplasm after castration and referred to the appearance of the basophils as "spongy" and "finely alveolar," respectively. Both groups of workers felt that the vacuole of the signet-ring cell arose as a separate vacuole in the cytoplasm with no perceptible relationship to the previous cytoplasmic subdivisions. Our observations indicate that the large vacuole of the signet-ring cell merely represents the product of the coalescence of smaller vesicular units.

It appears that two types of gonadotrophs may be distinguished by electron microscopy. The first type appears early after castration, and contains rounded, light vesicular nuclei and ovoid cytoplasmic vesicles. The second type, more prominent in the later castrates, contains dense, elongated or folded nuclei and irregularly shaped vesicles separated by cytoplasmic strands forming a bizarre cytoplasmic structure which resembles delicate filigree.

Purves and Griesbach (1952) have recently described two types of gonadotrophic cells in rat pituitaries. These two cell types can be differentiated by their response to physiological disturbances of the reproductive cycle and their response to experimental treatment. The authors show that one type of cell is located at the periphery of the gland, and one is more centrally located. The central gonadotrophs are prominent just prior to the onset of sexual maturity in the female and degranulate just prior to the first ovulation. Degranulation of these cells is promoted by estrogen injection which promotes storage in the peripheral gonadotrophs. Castration

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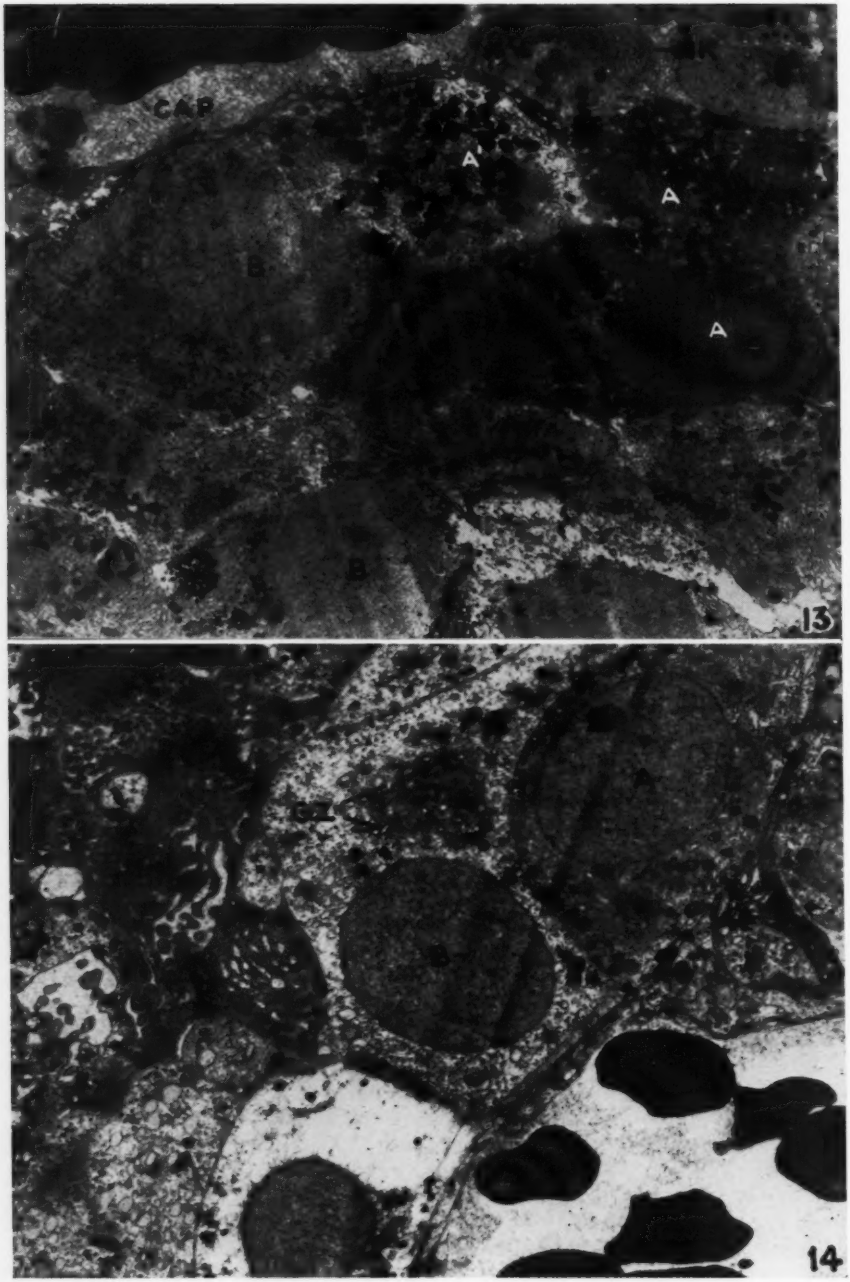
FIGS. 9–12. Seventy-five-day castrates. This group of figures illustrates cells which possess common features, distinguishing them from the cells illustrated in Figures 1–7. These cells are arranged to show increasing stages of vesiculation and granule loss resulting in a "signet-ring cell" quite different in appearance from those illustrated previously. The cells are characterized by a prominent Golgi zone (GZ), irregular or elongated, dense nuclei and bizarre, filigree-like cytoplasm. They are thought to represent cells producing L.H. Fig. 9, ×3500; Fig. 10, ×2500; Fig. 11, ×3500; Fig. 12, ×3000.


CAP
A
A
A
B
B
13
14

causes an increase in the number of both types of gonadotrophs. These authors suggest that the peripheral gonadotrophs may produce follicle-stimulating hormone (F.S.H.), and the central gonadotrophs may produce luteinizing hormone (L.H.). As further evidence to support this hypothesis, they point out that it is only several weeks after castration when the central gonadotrophs show an accumulation of glycoprotein that a strong lutenizing action is demonstrable in the pituitary.

In our electron micrographs the filigreed cells are not prominent in the female pituitary until several weeks after castration. It is, therefore, our belief that the later-appearing filigreed cells are the L.H. producers, and the earlier-appearing gonadotrophs are the F.S.H. producers. Thus, our independent observations would appear to support the concept of the existence of two gonadotrophic cell types as presented by Purves and Griesbach.

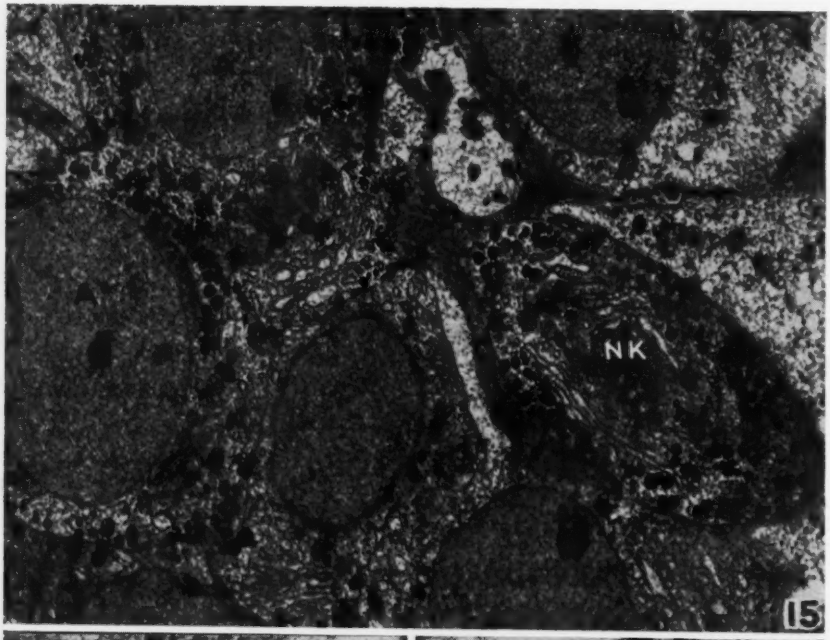
It has been shown by injections of pituitary extracts (Engle, 1929; Evans and Simpson, 1929) and by parabiosis experiments (Hill, 1933; Witschi and Levine, 1934) that the gonad-stimulating potency of the pituitary gland is greatly increased after castration. It seems logical to assume that the increased hormone content of the gonadotrophs is contained within the granules or the vesicles or both, as suggested by Severinghaus (1937).

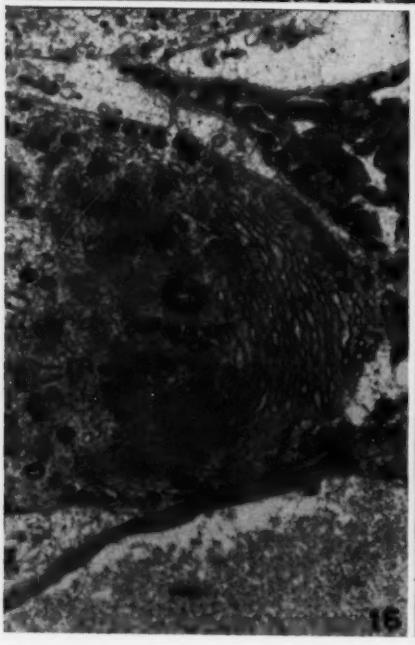
Certain speculations can be made about the granules and vesicles, their relationship to the PAS staining reaction in castration cells and their hormone potency. Purves and Griesbach (1951a) have pointed out that the PAS reaction in the granules in newly formed gonadotrophs after castration, though clearly positive, is not as intense as the gonadotroph of normal animals. On the other hand, the hyaline vacuole is only slightly pink in contrast to the intensely red-stained granules which surround it.

FIG. 13. Six-day castrate. This figure illustrates acidophil granules of two sizes. On the right are two heavily granulated acidophil cells (A), one showing a dense nucleus with a large nucleolus; the granules are smaller (maximal diameter, 350 mμ) and more rounded than the larger elliptical masses (maximal length, 600 mμ) seen in the acidophil (A'). For a further comparison in granule size, two basophils (B) with very fine granules (maximal diameter, 150 mμ) are seen in the field. Above are some more of these larger alpha particles in association with a circularly arranged canalicular apparatus which has been referred to as the "Nebenkern" (NK). ×5000.

FIG. 14. Seventy-five-day castrate. A degranulated basophil (B) is seen in the center of the field. The cytoplasm of this cell contains a few very fine granules, remnants of small vesicles and a compact Golgi apparatus located above the nucleus. In light microscopy such a cell would probably be classified as a chromophobe. The Golgi zone is denser than the adjacent cytoplasm and contains very fine vacuoles. This basophil is thought to be in a regenerative phase. It is not clear whether this cell represents a gonadotrophic or thyrotrophic basophil, but the latter is probable.

To the right of this cell is an acidophil (A) containing a few granules lined up along the cell membrane. Numerous light spherical mitochondria are seen chiefly below the nucleus. Delicate canaliculi make up the background. ×5000.


NK
15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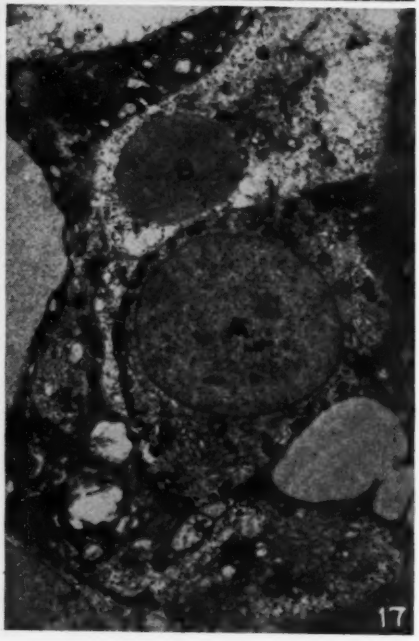
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We believe that the PAS reaction is more clearly related to the granules than to the vesicles in the castrate pituitary. The reasons for this belief are: 1) our own observations on PAS-stained material indicate that the PAS reaction is particularly strong within and around the Golgi apparatus where electron micrographs show the greatest granule accumulations (Figs. 4, 6, 9); 2) in electron micrographs of early castration cells it is seen that granules are present in sufficient quantities to be responsible for the positive reaction with PAS, even though the vesiculation of the cytoplasm is the most striking process taking place (Figs. 2 and 5); 3) examination of the signet-ring cells (Figs. 7) indicates that the large vacuole is surrounded by cytoplasm containing numerous granules, which could be responsible for the PAS reaction seen with the light microscope.

It is, therefore, our impression that the granules are responsible for the PAS-positive reaction of the gonadotrophs and that, as the granule content diminishes and vesicles are formed, some alteration takes place in the gonadotrophic substances which prevents intense PAS straining. However, it would seem that the vesicles must contain substances with gonadotrophic potency, for the granule content would not appear to be sufficient to account for the increased gonadotrophic potency of the castrate gland.

Canalicular Structures or Ergastoplasmic Filaments and Vesicles

Cytoplasmic filaments have been identified and described under a variety of names by numerous workers utilizing light or electron microscopy. These structures are sharply delineated by electron microscopy and have stimulated considerable interest. They have been called "endo-

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FIG. 15. Seventy-five-day castrate. On the right is a portion of an acidophil showing a coarse, circularly arranged canalicular apparatus in association with a few granules. In the center of the canalicular structure is a dense mass of osmiophilic material. This arrangement of the cytoplasmic filaments about a central mass or core constitutes the "Nebenkern" (NK).

On the left are two other acidophils (A) containing large nuclei surrounded by a small rim of cytoplasm in which are seen a few canaliculi, mitochondria and granules. ×6000.

FIG. 16. Seventy-five-day castrate. A portion of an acidophil showing coarsened canalicular structures in association with a few granules is illustrated. Below, the canaliculi are finer and more delicate, and to the right they are coarser and show irregular saccular dilatations forming a chainlike structure. Large pale spherical mitochondria (m) are seen to the left of the canaliculi. ×6500.

FIG. 17. Seventy-five-day castrate. Another acidophil (A) with a peripheral rim of granules is seen in the center. The nucleus is relatively large and the nuclear membrane is sharply defined. Below the nucleus are a few spherical mitochondria which show irregular inner densities.

Above the acidophil is a degranulated basophil (B) with a few fine granules and frayed-appearing mitochondria. The nuclear membrane is indistinct. ×4500.

plasmic reticulum" by Palade and Porter (1952a), "lamellae" by Dalton *et al.* (1950), cytoplasmic "double membranes" by Sjöstrand (1953), "filamentous structures" by Braunsteiner *et al.* (1953), and "basophilic cytoplasmic structures" by Oberling *et al.* (1953).

Such structures were seen with the light microscope as early as 1869 (see Bowen, 1929 and Kirkman, 1937 for a review of the literature). In certain acidophils, it was noted that these fine filaments were frequently arranged in concentric whorls radiating outward from a central core containing granules or irregular masses of eosinophilic material. This circular arrangement of the cytoplasmic filaments about a central mass or core was called the "Nebenkern."

Some of the early workers believed that the cytoplasmic filaments were artefacts produced by the action of acid-fixing agents upon the cytoplasm, but Saguchi (1920), Opie (1928), and Kirkman recognized the possibility that they were preformed structures rendered visible by the action of acid fixatives. However, evidence contributed by electron microscopy indicates that the double-layered canalicular structures are true cytoplasmic constituents present in most cells. Variations in form and prominence appear to be related both to the cell type and its functional state.

In certain acidophils of the anterior pituitary gland, such structures are sharply delineated (Rinehart and Farquhar, 1953). A variant of this structure appears to be present in the basophils. In the normal rat, segments of similar double-membraned structures are seen in certain of the nonvesiculated basophils. In the earlier phases of vesiculation there appear to be transitions between slightly expanded canaliculi and vesicles (Fig. 2). Not infrequently, in acidophils the double-layered canaliculi show periodic expansions and constrictions producing a chainlike structure (Figs. 2, 16). It seems likely that a basically similar canalicular structure exists in the basophils. The vesicles of the basophils may represent expansions of the double-layered canaliculi between points of constriction (Fig. 2) and may serve to store hormonal products. No doubt, further studies will clarify the nature and function of this remarkable cytoplasmic structure.

Golgi Apparatus

Severinghaus (1933) has described the Golgi apparatus of the acidophil as a "basket-like network closely applied to the nucleus" and the Golgi apparatus of the basophil as "a compact spherical mass lying free in the cytoplasm." We have noted that in ultrathin sections seen with the electron microscope, the Golgi apparatus of the acidophils and basophils appears generally similar; it is seen as a more or less differentiated zone of cytoplasm usually bordered by a chain of clear vacuoles. The size and form of the structure varies considerably with the physiologic state of the cell. Mitochondria are often seen in intimate relationship to the Golgi apparatus

giving the area the appearance of a center of physiologic activity. In certain degranulated cells, the Golgi apparatus appears quite dense and compact (Fig. 14). In the fully granulated cell the Golgi apparatus is inconspicuous or not visible.

Enlargement of the Golgi apparatus accompanying basophil hypertrophy has often been described as a prominent feature of the early castrate. In electron micrographs of the pituitaries of castrates the Golgi apparatus is enlarged six days after castration, when it has the appearance of a fine beadlike vacuolar chain surrounding an area of cytoplasm which is denser than the remainder of the cytoplasm (Fig. 1). The cytoplasm in and around the Golgi apparatus contains a greater concentration of granules and greater numbers of mitochondria than the surrounding cytoplasm. At 10 and 20 days after castration the Golgi apparatus of the predominating castration cells appears somewhat larger with additional complication of its vacuolar chain (Figs. 4 and 5). In the larger multivesiculated cells of the thirty-five and seventy-five-day castrates, the Golgi body is still larger than seen in the basophils of the controls but is smaller than in the gonadotrophs characteristic of the earlier castrates. At this stage the Golgi often occupies the only area of the cytoplasm which is not vesiculated (Figs. 6, 9 and 10).

Severinghaus (1933) and Wolfe and Brown (1942) associated enlargement of the Golgi apparatus with elaboration of secretion and an increase of mitochondria with release of secretion or degranulation. However, as Foster (1942) points out, in the endocrine glands it is very difficult to separate the two activities of secretion production and secretion release, and it is possible that the Golgi apparatus may function in one or both of these processes. Our observations on the pituitaries of castrates and those of the controls would seem to indicate that the enlargement of the Golgi body may be associated with both of these processes.

Mitochondria

Recent electron microscopic studies of Palade (1952c, 1953) and Sjöstrand (1953) have shown that the mitochondria possess a limiting membrane and a system of internal ridges which probably serves to increase the surface area for the metabolic functions in which these structures participate. Palade (1953) has suggested that these internal ridges may serve as a site for the localization of enzymatic chains.

Our studies on the rat anterior pituitary indicate that the size, shape, density and internal organization of the mitochondria may vary considerably with the functional activity of the cell.

In control female rats we have seen a wide variation in the appearance of the mitochondria. In the acidophils, which appear to be in a chromophobic or relatively degranulated phase, the mitochondria are seen to be

less prominent, less dense and, at times, somewhat frayed in appearance as compared with cells which are resuming functional activity and accumulating or releasing granules. In the degranulated basophils the mitochondria appear about the same as in the degranulated acidophils but, as the cells acquire granules, the mitochondria become more dense until, in the vesiculated forms, they are intensely osmiophilic and appear of a uniform density. In this stage they are most often seen as short rods or spheres. However, filaments and rods of various lengths are seen in both acidophils and basophils with varying stages of the secretory activity.

In the castrate pituitaries several features of mitochondrial variation are noteworthy. The first is the prominence of the large spherical mitochondria of light density found in the partially degranulated acidophils. The similar appearance of the mitochondria in many acidophils in the castrate pituitaries is presumably due to the fact that the cells are seen in similar stages (of lowered functional activity) as evidenced by other common cytologic features, rather than due to inadequate fixation or "aging" of the mitochondria, as suggested by Weiss and Lansing (1953).

Other features of note are the number, distribution and form of the mitochondria in the enlarged gonadotrophs of castration. The numbers of mitochondria appear to be increased in all periods after castration. As Reese, Koneff and Wainman (1943) have pointed out, there is a definite concentration of the mitochondria in the region of the Golgi apparatus from which they radiate into the surrounding cytoplasm. Their form varies with the interval after castration. In the early phases filaments and rods are frequently seen, but as the interval after castration increases, short rods and spheres predominate.

In short, indications are that mitochondria show variations in size, form, density and internal structure with varying stages and degrees of functional activity, although data are not sufficient to date to clearly establish specific variations of mitochondrial appearance with specific stages of secretory activity.

Effects of Castration on the Acidophils

Observations on the effects of castration on the acidophils have been confusing and conflicting and have ranged from reports of increased numbers of acidophils to reports of a decrease in their size and staining capacity (see Severinghaus, 1937, for a review.). Our findings seem to be in agreement with those of Severinghaus who noted a progressive decrease in the acidophil granulation up to 46 days after castration with increased granulation evident thereafter. One gains the impression that there has been a temporary suppression in the formation of granules, while release of acidophil secretory products has continued. This conclusion is reached 1) because of the frequency with which cells having only a rim of granules

are seen, and 2) because of the lack of cytologic evidence that these cells are accumulating granules. The small amount of cytoplasm and the inconspicuous or absent Golgi apparatus would indicate depressed functional activity.

What is the significance of the acidophil cells which contain the larger elliptical granules? Two possibilities come to mind. The first is that these large masses may represent merely a step in the synthesis of acidophil secretory products—either a larger particle which is subsequently broken down into granules for release or a product of condensation of smaller granules. The second possibility is that the cells containing the large ovoid particles could represent a different cell type. It is possible that the more numerous cells with the smaller spherical granules are producing growth hormone, whereas the larger masses could characterize cells producing one of the other pituitary hormones such as ACTH or lactogenic (luteotrophic, mammotrophic) hormone. (See Herlant (1952) for a review of the literature regarding the site of production of these two hormones.)

Purves and Griesbach (1952) have recently described two types of acidophils in the rat pituitary which were differentiated by their responses to experimental treatment. One type is thought to be responsible for growth hormone and is described as being columnar in shape and arranged radially around sinusoids or in palisades along connective tissue septa. The other type, suggested to be lactogenic and luteotrophic in function, they found to be located on the interior of cell cords. In castrated animals and in animals treated with estrogen, these authors noted that the second type is degranulated and the first more prominently displayed. In thyroxine deficiency the first type is degranulated; the second type persists. It is possible that the difference in granule size in certain acidophils we have noted with the electron microscope will serve to differentiate the two types of alpha cells postulated by Purves and Griesbach. However, further observations on control and experimental material will be necessary before additional interpretations can be made regarding the nature and functional significance of the cells which are found by electron microscopy to contain the large elliptical granules.

The surprisingly fine cytologic detail revealed in examination of ultrathin sections by electron microscopy appears clearly to offer much promise in histologic research. With specific reference to the anterior pituitary gland, this method, particularly when combined with histochemical methods, should aid in clarification of the intriguing and baffling problems of cell structure and function.

Studies currently in progress indicate that the vesicular cells of thyroidectomy, as revealed by electron microscopy, exhibit clear morphologic features differentiating them from those seen following castration. In a subsequent report, we will present our observations on "thyrotrophs"

which support the concept of Purves and Griesbach and Halmi that gonadotrophs and thyrotrophs are separate cell types.

SUMMARY

Adult female rats of the Sprague-Dawley strain were castrated at two months of age and sacrificed at intervals of 6, 10, 20, 35 and 75 days thereafter. Segments of the pituitary were fixed in buffered osmic acid and embedded in methacrylate; ultrathin sections were then prepared for examination with an RCA electron microscope.

Certain gonadotrophic cells were seen to enlarge as early as six days following castration and to undergo a regular sequential change in structure as the days after castration increased. These gonadotrophs showed an increase in granular and mitochondrial content, enlargement of the Golgi apparatus and pronounced vesiculation of the cytoplasm. As the period after castration increased, the average size of the vesicles enlarged by coalescence of small rounded units into larger, more irregular bodies. At the same time the granule content was seen to diminish. In the late stages (thirty-five- and seventy-five-day castrates) many of these cells were seen with just a fringe of cytoplasm surrounding one or several large vesicles.

Other basophils, quite different in appearance from those described above, were seen in the pituitaries of thirty-five- and seventy-five-day castrates. These cells were also greatly enlarged and showed varying degrees of vesiculation but were characterized by denser, more irregular nuclei and bizarre cytoplasmic formations resembling filigree. Such cells are illustrated, and the possibility of their being another subvariety of gonadotrophic cell is suggested. It is thought that these filigreed gonadotrophs may be producing L.H. and that the previously described cells may be producing F.S.H. Evidence supporting this view is presented.

Acidophils which appear to be of two types were also noted. One cell contained smaller rounded granules; the other contained larger elliptical granular masses. These cells are illustrated, and their possible significance is discussed.

Variations observed in the mitochondria, Golgi apparatus and canalicular apparatus (ergastoplasmic filaments) are described and discussed.

The authors feel that more studies of this type with the electron microscope, correlated with histochemical studies, will serve to clarify cytologic alterations accompanying secretion in the various cell types of the anterior pituitary and may aid in establishing the presence of distinctive cellular subvarieties responsible for the production of each of the specific pituitary hormones.

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THE VALUE OF A HIGH PROTEIN DIET IN THE PREVENTION OF ANEMIA IN HYPOPHYSECTOMIZED ADULT FEMALE RATS BY MEANS OF THYROXINE AND ANDROGEN THERAPY[1]

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HYPOPHYSECTOMY has induced an anemia in every animal that has been investigated, including the human being (see reviews by Gordon and Charipper, 1947, and Daughaday, Williams and Daland, 1948). Thyroxine therapy in hypophysectomized rats has prevented the decrease in erythrocyte count but did not maintain the hemoglobin at normal levels (Meyer, Thewlis and Rusch, 1940; Vollmer, Gordon and Charipper, 1942; Crafts, 1946a). Androgen therapy in hypophysectomized rats gave similar results, i.e., the erythrocyte count was maintained at normal levels but the hemoglobin decreased (Vollmer and Gordon, 1941; Vollmer, Gordon and Charipper, 1942; Crafts, 1946b). Crafts (1949) prevented all aspects of the anemia which is induced by hypophysectomy in adult female rats by daily injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate combined with a high protein diet. These data indicated that faulty protein metabolism might play a role as a cause of the anemia in hypophysectomized rats. Growth hormone therapy, however, has been shown to be ineffective in preventing anemia in hypophysectomized rats (Meyer, Stewart, Thewlis and Rusch, 1937; Van Dyke, Simpson, Garcia and Evans, 1952; Crafts, 1953a).

The question arises as to whether the beneficial effects obtained with the thyroxine, androgen, and high protein diet therapy in hypophysectomized animals mentioned above (Crafts, 1949) were due to the thyroxine and androgen therapy only. The present experiment was done to clarify the value of a high protein diet in prevention of anemia in hypophysectomized rats by means of thyroxine and androgen therapy.

METHODS

Adult female rats of the Wistar strain, 3–4 months of age, were used in this experiment. All rats, except those receiving the protein diet, were fed standard Purina chow ad

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[2] With the technical assistance of Dorothy S. Turnbull and Nancy H. Lancaster.

libitum supplemented once a week with lettuce. The high protein diet was obtained by combining the standard Purina chow diet with powdered casein in equal quantities. This provided a 62.5% protein diet. This is a high content of protein but is the same as used in the previous experiment (Crafts, 1949). Completeness of the hypophysectomies was verified by examination of the sella turcica under a binocular dissecting microscope. Blood was obtained by anesthetizing the rat with ether and cutting the tail. Five or 6 drops of blood were placed in a small vial containing a minute amount of heparin. Erythrocyte counts were done in duplicate using U. S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made with a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 r.p.m.

RESULTS

This experiment was done on 44 adult female rats. Ten served as con-

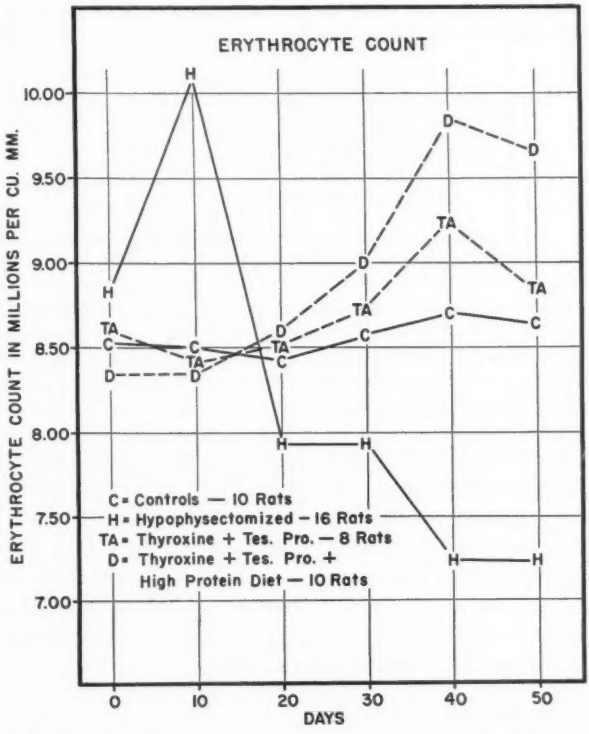

FIG. 1. The effects of hypophysectomy only, hypophysectomy followed by daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate, and of hypophysectomy followed by a high protein diet in addition to daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate on the erythrocyte count of adult female rats.

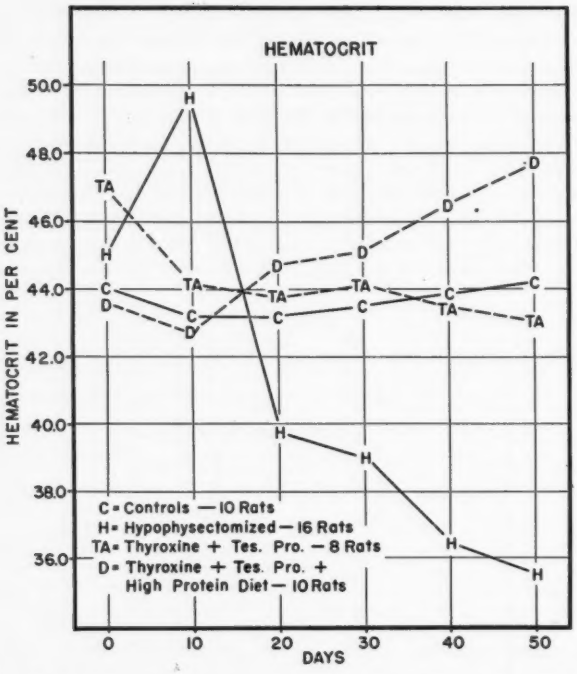

FIG. 2. The effects of hypophysectomy only, hypophysectomy followed by daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate, and of hypophysectomy followed by a high protein diet in addition to daliy subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate on the hematocrit reading of adult female rats.

trols, 16 were hypophysectomized only, 8 were hypophysectomized and given daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate, and 10 were hypophysectomized and fed a high protein diet in addition to receiving the same amount of thyroxine and testosterone propionate. Blood was studied at 10 day intervals and the experiment was terminated on the 50th day after hypophysectomy. The results on erythrocyte count, hematocrit reading, and on hemoglobin levels are shown in Figures 1, 2, and 3 respectively.

Normal Untreated Controls

The normal untreated control rats showed no significant changes during the 50 days of observation. The blood picture in these rats before the experiment started was as follows:—erythrocyte count, 8.50 ± 0.70[3] million

[3] Standard Deviation.

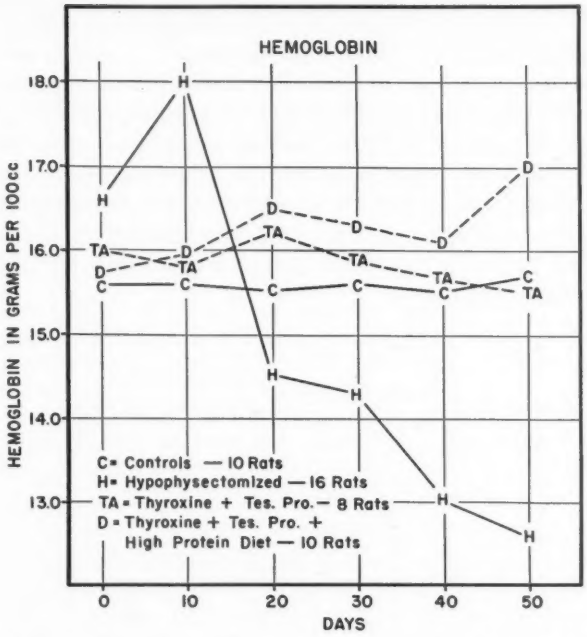

FIG. 3. The effects of hypophysectomy only, hypophysectomy followed by daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate, and of hypophysectomy followed by a high protein diet in addition to daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate on the hemoglobin level of adult female rats.

cells per cu. mm.; hematocrit reading, $44.0 \pm 3.7\%$; and hemoglobin level, 15.6 ± 1.1 gm. per 100 cc. At the end of the experiment, 50 days later, these untreated control rats exhibited the following blood picture:—erythrocyte count, 8.63 ± 0.90 million cells per cu. mm.; hematocrit reading, $44.4 \pm 2.2\%$; and hemoglobin level, 15.7 ± 0.8 gm. per 100 cc. The body weight of the untreated control rats increased from an average of 188 gm. before the experiment started to 228 gm. on the 50th day of the experiment.

Hypophysectomy

Removal of the hypophysis induced the usual anemia. The erythrocyte count decreased from 8.78 ± 0.4 million cells per cu. mm. before hypophysectomy to 7.23 ± 0.6 million cells 50 days later; the hematocrit reading, from $45.0 \pm 2.6\%$ to $35.5 \pm 2.7\%$; and the hemoglobin, from 16.8 ± 0.7 gm. per 100 cc. of blood to 12.6 ± 0.8 gm. These hypophysectomized animals decreased in weight from an average of 204 gm. before hypophysectomy to 183 gm. on day 50 of the study.

Hypophysectomy followed by Thyroxine and Androgen Therapy

Daily subcutaneous injections of 0.005 mg. of thyroxine[4] and 1.0 mg. of testosterone propionate[5] prevented the anemia which is induced by hypophysectomy. Fifty days after hypophysectomy the erythrocyte count in these treated rats was 8.83 ± 0.63 million cells per cu. mm. (normal controls—8.63 ± 0.90), the hematocrit reading was $43.2 \pm 1.5\%$ (normal controls—44.4 ± 2.2), and the hemoglobin level was 15.6 ± 0.4 gm. per 100 cc. of blood (normal controls—15.7 ± 0.8). These treated hypophysectomized rats decreased in body weight from a pre-operative average of 202 gm. to 180 gm. 50 days later.

Hypophysectomy followed by Therapy with Thyroxine, Androgen, and High Protein Diet

A high protein diet in addition to daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate in hypophysectomized rats caused increases in the erythrocyte count, hematocrit reading, and hemoglobin levels. This treatment induced an increase in erythrocyte count from 8.33 ± 0.7 million cells per cu. mm. before hypophysectomy to 9.68 ± 0.4 million cells on the 50th day of the experiment; an increase in hematocrit reading from $43.7 \pm 3.0\%$ to $47.6 \pm 2.5\%$; and an increase in hemoglobin level from 15.6 ± 0.9 gm. per 100 cc. to 17.0 ± 0.6 gm. These increases are statistically significant with "P" values[6] of <0.01, <0.01, and <0.001 respectively. The body weight of these animals averaged 186 gm. before hypophysectomy. After 50 days of treatment the body weight averaged 169 gm.

DISCUSSION

As noted in the introduction, anemia of hypophysectomized rats has been prevented by a combined therapy of thyroxine, androgen, and a high protein diet (Crafts, 1949). Absolutely negative results in the prevention of anemia in hypophysectomized rats with growth hormone therapy (Meyer, Stewart, Thewlis and Rusch, 1937; Van Dyke, Simpson, Garcia and Evans, 1952; Crafts, 1953a) cast suspicion on the value of the high protein diet in the above experiment. Data presented in this paper, however, show that anemia in hypophysectomized rats can be prevented by thyroxine and androgen when the rats are on a regular diet, but that better results can be obtained if a high protein diet is added to the therapy. Furthermore, a diet deficient in protein is known to induce an anemia (Cartwright, 1947).

[4] The thyroxine was generously furnished by the Organon Company, Inc.

[5] The testosterone propionate was generously furnished by Schering Corporation.

[6] P = Fisher's "t" test of significance.

Of equal interest is the report that combined thyroidectomy and adrenalectomy induces an anemia in rats which mimics the anemia which follows removal of the hypophysis (Crafts, 1953b).

The evidence mentioned above substantiates the hypothesis that the anemia which follows hypophysectomy is due to the faulty metabolism which exists in hypophysectomized animals. Every therapeutic measure that has been shown to have a preventive effect on anemia in hypophysectomized animals, with the exception of cobalt therapy (Crafts, 1952; Garcia, Van Dyke and Berlin, 1952) and orally administered pituitary extracts (Flaks, Himmel and Zlotnik, 1938; Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams and Evans, 1953), has contained a substance known to have an effect on metabolism. Such substances, shown to be partially or completely effective in preventing anemia in hypophysectomized rats, have been thyroxine (Meyer, Thewlis and Rusch, 1940; Vollmer, Gordon and Charipper, 1942; Crafts, 1946a), a combination of thyroxine, iron and copper (Crafts, 1946a), androgen, either directly or induced by gonadotropic hormone (Vollmer and Gordon, 1941; Vollmer, Gordon and Charipper, 1942; Crafts, 1946b), a combination of thyroxine, androgen, and a high protein diet (Crafts, 1949), and adrenocorticotropic hormone (Garcia, Van Dyke Huff, Elmlinger and Oda, 1951).

The two exception noted above; i.e., cobalt and orally administered pituitary extracts, demand explanation. Cobalt therapy seems to induce erythropoiesis by interfering with internal respiration of the tissues. This produces a state of anoxia which, in turn, stimulates the bone marrow (Warren, Schubmehl, and Wood, 1944; Orten and Bucciero, 1948; Bucciero and Orten, 1949; Wesley, 1951). In addition, there is evidence that cobalt enhances the utilization of iron reserves in the formation of hemoglobin (Kato and Iob, 1940; Greenberg, Ashenbrucker, Lauritsen and Wintrobe, 1947; Wintrobe, Greenberg, Humphreys, Ashenbrucker, Worth and Kramer, 1947; Wintrobe, Grinstein, Dubash, Humphreys, Ashenbrucker and Worth, 1947). It is difficult to imagine, however, that the anemia in hypophysectomized animals is due to a lack of cobalt, although this point should be investigated. Beef pituitary extract, which was free of thyrotropic, gonadotropic, and growth hormones has been shown to be effective in preventing anemia in hypophysectomized rats (Flaks, Himmel and Zlotnik, 1938). Similar results have been reported in hypophysectomized rats by Contopoulos and co-workers (1953) with sheep pituitary extract. These groups of workers both agree that there is an "erythropoietic factor" released by the hypophysis. It should be noted, however, that these extracts were administered orally. Assuming that such an "erythropoietic factor" would be similar chemically to other factors released by the an-

terior lobe of the hypophysis, it is difficult to see why they are effective when administered by this route.

The present experiment has shown that anemia in hypophysectomized rats can be prevented by combined therapy with thyroxine and androgen, and that a high protein diet in conjunction with the above therapy is beneficial. Evidence is gradually accumulating which indicates that the anemia which is induced by hypophysectomy is due to faulty metabolism and that there is no necessity for an "erythropoietic hormone" secreted by the hypophysis as proposed by Flaks and co-workers (1938) and by Contopoulos and co-workers (1953).

SUMMARY

In order to determine the value of a high protein diet in the treatment of anemia in hypophysectomized rats with thyroxine and androgen, the blood pictures of four groups of adult female rats of the Wistar strain were studied. These 4 groups were:—(1) normal controls, (2) hypophysectomized only, (3) hypophysectomized followed by daily subcutaneous injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate, and (4) hypophysectomized followed by a high protein diet in addition to the same doses of hormones as were given to group 3.

No changes occurred in the untreated normal control rats. On the 50th day of the experiment, the last day, these rats had an erythrocyte count of 8.63 million cells per cu. mm., a hematocrit reading of 44.4%, and a hemoglobin level of 15.7 gm. per 100 cc of blood.

Hypophysectomy induced the usual anemia. These rats, 50 days after hypophysectomy, had an erythrocyte count of 7.23 million cells per cu. mm., a hematocrit reading of 35.5%, and a hemoglobin level of 12.6 gm. per 100 cc. of blood.

The hypophysectomized rats which received daily injections of thyroxine and testosterone propionate for 50 days showed no anemia. On the 50th day of treatment, the erythrocyte count was 8.83 million cells per cu. mm., the hematocrit reading was 43.2%, and the hemoglobin level was 15.6 gm. per 100 cc.

Treatment of hypophysectomized rats with a high protein diet for 50 days, in addition to the thyroxine and testosterone propionate, induced a rise in the erythrocyte count to 9.68 million cells per cu. mm., a rise in hematocrit reading to 47.6%, and a rise in hemoglobin level to 17.0 gm. per 100 cc.

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PREVENTION OF EXPERIMENTAL DIABETES WITH ADRENAL STEROIDS[1]

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AFTER subtotal pancreatectomy in white rats (removal of approximately 95% of the pancreas), blood sugar level remains normal for a few weeks, and then diabetes develops progressively (Foglia, 1944). The administration of estrogens during six months after pancreatectomy has a preventing action on the onset of diabetes (Foglia, Schuster and Rodriguez, 1947; Lewis, Foglia and Rodriguez, 1950; Rodriquez, 1951).

In alloxan diabetic rats recovery to normal glycemia can be obtained by estrogen treatment and especially by the combined treatment of estrogen and insulin (Rodriguez, 1953).

The experiments here reported show that the prolonged administration of small amounts of certain adrenal corticoids has also a marked protective action on the onset of diabetes in partially pancreatectomized rats.

With large amounts of adrenal steroids there is an initial increase in the incidence of diabetes which is followed by a secondary decrease.

METHODS

Female white rats from the Institute strain were used. When they had reached 80 to 100 gm. body weight, the ovaries and 95% of the pancreas were removed. A week later treatment was started, consisting of one daily subcutaneous injection of the steroid mixed with 25 mg. of lactose suspended in 0.5 ml. saline (0.9% NaCl) solution. The controls received 25 mg. lactose in saline. Treatment was continued for six months. The animals were fed "ad libitum" the stock diet of our Institute (Lewis, Foglia and Rodriguez, 1950). The body weight and the blood sugar were determined monthly and an animal was considered to be diabetic when the blood sugar was above 150 mg. % after a seven hour fast. At the end of the experiment the animals were sacrificed, the organs weighed and the pancreatic tissue remaining was fixed in Helly solution and stained with Gomori's chromic hematoxylin.

RESULTS

Table 1 shows that desoxycorticosterone acetate, at a dose of 100 μg. per

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[2] Fellow of the E. Santamarina Foundation.

TABLE 1. EFFECT OF DIFFERENT ADRENAL STEROIDS, INJECTED DAILY DURING SIX MONTHS, ON THE INCIDENCE OF DIABETES IN PARTIALLY PANCREATECTOMIZED RATS

Drug	Daily dose per rat, μg.	No. of rats	Diabetes (percentage)					
			Months					
			1	2	3	4	5	6
Lactose (controls)	25 mg.	18	0	11	22	33	55	77
Desoxycorticosterone	100	12	0	16	16	33	50	66
Compound A	50	16	0	47	37	25	25	37
Compound E	150	17	23	58	47	47	35	35
Compound E	50	17	0	6	6	17	11	11
Compound F	50	14	0	7	14	28	28	14

rat per day has no significant influence on the onset of diabetes in our partially depancreatized rats.

Compound A, 50 μg. per rat per day and Compound E, 150 μg. per day, increased the incidence of diabetes during the first three months after partial pancreatectomy; afterwards, no rats became diabetic and some of the diabetic animals returned to normal blood sugar levels and remained free of diabetes, after six months of treatment.

When 50 μg. of either compound E or F/rat/day were administered the number of diabetic rats was not increased during the first three months of treatment, but a marked decrease in the incidence of diabetes was observed at the end of the six months' observation period.

The growth of the non-diabetic animals injected with the different corticoids was similar to the non-diabetic controls. The autopsies, at the end of the experiment, showed that the small amounts of corticoids injected daily had no statistically significant action upon the weight of the adrenals, thymus, thyroids and hypophysis. The weight of the uterus of the animals treated with compounds E and F were similar to the controls (non-diabetic partially pancreatectomized-ovariectomized rats). The animals injected with compound A or desoxycorticosterone had a definite increase in the uterine weight (Table 2).

TABLE 2. WEIGHT OF ORGANS (IN MG. PER 100 GM. BODY WEIGHT) OF FEMALE CASTRATED RATS INJECTED DIFFERENT ADRENAL STEROIDS DAILY DURING SIX MONTHS AFTER REMOVAL OF 95% OF THE PANCREAS

Organ	Lactose 25 mg.	Desoxy-cortico. 100 μg.	Comp. A. 50 μg.	Comp. E. 150 μg.	Comp. E. 50 μg.	Comp. F. 50 μg.
Hypophysis	5.2	6.2	7.9	6.9	6.0	5.0
Adrenals	22.4	18.8	29.6	24.0	23.1	19.9
Thymus	99	137	99	136	85	123
Thyroids	9.8	8.6	12.1	10.6	11.6	9.1
Uterus	20	95	130	27	29	20
Body weight when killed (gm.)	172	180	179	172	176	194

The remaining pancreatic tissue was examined histologically by one of the authors, Dr. A. F. Cardeza. Animals treated with compounds A, E and F, which did not become diabetic showed marked hyperplasia of the insular tissue, with hypertrophy of the islets. The beta cells appeared normal with well preserved and normally stained granules.

SUMMARY

The effects of adrenal steroids (given daily for six months), on the incidence of diabetes in partially pancreatectomized-ovariectomized rats were studied. The results were:

(1) No action of desoxycorticosterone acetate in the dose of 100 μg./rat/day.

(2) The administration of compounds A, E and F, in doses of 50 μg. to 150 μg./rat/day, markedly decreased the number of diabetic rats at the end of six months.

(3) With the higher doses of compound A (50 μg./rat/day) and compound E (150 μg./rat/day) the action was diphasic, i.e. an initial increase and a secondary decrease in the incidence of diabetes. With a small dose of compound E, only the second (preventive) phase was observed.

(4) The animals in which the protective action of adrenal steroids was observed, showed a marked hypertrophy and hyperplasia of the islets of Langerhans of the pancreas.

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IN VITRO BIOASSAY FOR THE MELANOCYTE STIMULATING HORMONE[1]

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IN 1919 Atwell showed that extracts from mammalian pituitary glands darkened the skin of hypophysectomized tadpoles. Since then the active principle, considered a hormone, has been known as intermedin, melanophore dilating principle, melanophore hormone, etc., with reference to its source in the intermediate lobe of the pituitary gland or its site of action, the melanophores of amphibia. Because the term *melanocyte* (Fitzpatrick and Lerner, 1953, Gordon, 1953) recently was adopted as the name for the melanin forming cell in all animals, and because mammalian as well as amphibian melanocytes are affected by the pigment-cell activating principle of the pituitary gland, the latter has been designated as *melanocyte stimulating hormone* (MSH).

Most of the early methods for determining MSH consisted of observing *in vivo* darkening of frogs or fish injected with the hormone. Recently Friedin, Fishbein, and Hisaw (1948) described an *in vitro* assay in which the darkening of isolated frog skin could be measured quantitatively by determining the increase in light absorption by means of a photoelectric photometer. The latter method has been modified so that the darkening of isolated frog skin can be readily determined quantitatively by the use of photoelectric reflection measurements. This bioassay can be used to determine MSH in body fluids and tissue extracts.

METHOD

As test materials the skin of *Rana pipiens* frogs[2] (30–50 grams) were used. Skin from the thighs and legs was removed and cleaned of large vessels. Four pieces of skin could be obtained from each frog, one piece from each thigh and one from each lower leg. The skin samples were placed in a frame consisting of two rings, Figure 1. The outer ring was made of bakelite, and the inner of aluminum. Notches and holes in the bakelite frame were made in order to prevent the accumulation of air bubbles and to allow free

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[2] Supplied from E. G. Steinhilber & Co., Oshkosh, Wisconsin.

circulation of the test solution. Excess skin was cut off, and the frame with the surface of the skin downward was placed in a 50 ml. beaker containing 20 ml. of frog Ringer's solution (NaCl 6.50 gm./l., KCl 0.14/l., $CaCl_2$ 0.12 gm./l, $NaHCO_3$ 0.20 gm./l.).

A photoelectric reflection meter[3] was used to measure light reflection from the skin. The search unit was placed on a table with its opening pointing upward, and the beaker containing the frame with the skin was placed on top of it so that the skin in the frame fitted over the search unit opening. Although the frame was made to fit the search unit opening of the reflection meter, the light from this source was not of equal intensity throughout the field. For this reason a mark was put both on the frame and on the search unit, and the beaker was placed to conform to these markings so that the position of the skin with respect to the search unit opening was constant throughout every reading. Zero suppressor control knobs were turned completely clockwise, and the sensitivity

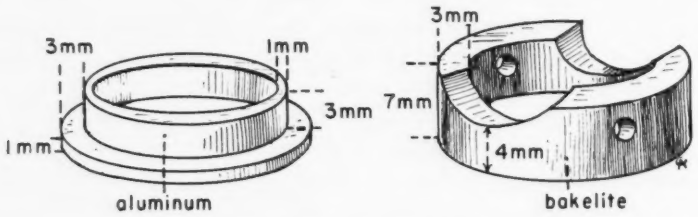

FIG. 1. Frame for frog skin.

control knobs were adjusted to 70 for the white enamel disc when a red filter was used. The enamel disc and the red filter accompanied the reflection meter. For the reading of frog skin no filter was used. Room light must be kept constant during the assay.

After the skin was put into the 20 ml. of Ringer's solution, an increase in reflection occurred gradually, because of the spontaneous lightening in color of the melanocytes. After about one hour, lightening of the melanocytes reached a maximum. During this time the Ringer's solution was changed once. The reading at the end of one hour was taken as the baseline value. If the reflectance were more than 100 or less than 70, the skin was discarded in order to approximate constancy of color among the skin samples. If 0.1 or 0.2 ml. of solution containing sufficient MSH were added to the Ringer's solution after the maximum reading was obtained, a rapid decrease of reflection resulted. By replacing the MSH solution with fresh Ringer's solution, the reflection increased again. And if MSH solution were added again, the reflection decreased as previously.

As there is no standard unit of the melanocyte stimulating hormone, the effect produced by 0.04 μm. of the following preparation made in this laboratory was tentatively defined as one unit of MSH. Two grams of beef posterior pituitary powder prepared by the Armour Laboratory was mixed with 200 ml. of distilled water, shaken for 1 hour with a mechanical shaker at room temperature, and centrifuged at approximately 12,000 r.p.m. The supernatant liquid was lyophilized and the resulting yellowish white powder weighed 490 mg. This powder, which has been kept in a vacuum desiccator, was used for the preparation of the standard solution.

[3] Photovolt Photoelectric Reflection Meter, Mod. 610, made by Photobolt Corporation, 95 Madison Ave., New York 16, N. Y.

ASSAY RESULTS

Eight series of skin samples were treated with Ringer's solution containing unknown amounts of MSH ranging from 0.125 unit per ml. to 2 units per ml. Sixty minutes later the decrease of reflection from the baseline reading (Δ_1) was recorded. All the samples were then washed for 30 minutes with fresh Ringer's solution and immersed in Ringer's solution containing 1.0 unit per ml. of standard MSH. Sixty minutes later another reading was taken, and the difference from the original baseline reading was recorded as Δ_2. For example, with one frog skin, the baseline reading was 82. One hour after 2 units per ml. of MSH was added, the reading was 33.

TABLE 1. VALUES OF Δ_1/Δ_2 OBTAINED WITH VARIOUS CONCENTRATIONS OF MSH

0.125U./ml.	0.25U./ml.	0.5U/ml.	1.0U/ml.	2.0U/ml.
0.35	0.59	0.76	1.18	1.23
0.39	0.31	0.81	0.96	1.26
0.24	0.57	0.90	0.94	1.32
0.24	0.58	0.79	1.12	1.13
0.15	0.38	0.63	1.00	1.34
0.21	0.49	0.69	0.95	1.21
0.13	0.54	0.63	0.90	1.31
0.30	0.61	0.78	0.83	1.20

Then the skin was washed, and 30 minutes later 1 unit per ml. of MSH was added. After one hour the reading was 42. In the case, $\Delta_1 = 82 - 33 = 49$; $\Delta_2 = 82 - 42 = 40$. When the ratio Δ_1/Δ_2 was plotted against the logarithm of the concentration of MSH (log C) a fairly good linear relationship was obtained. All values of Δ_1/Δ_2 for each experiment are shown in Table 1.

These results may be expressed by the linear regression equation

$$\Delta_1/\Delta_2 = 1.01 + 0.83 \times \log C$$

Standard error of estimate, $s_{yx} = \pm 0.093$.

The analysis of variance, with regression, is shown in Table 2.

Although in a strict sense this regression equation may be used only for predicting the responses Δ_1/Δ_2 with a known amount, it may be used in reverse to obtain an approximation of an unknown concentration, given Δ_1/Δ_2. If a two-skin assay is performed on a sample of unknown concentration, and the mean of the two Δ_1/Δ_2 values is used to enter the regression equation, the logarithm of concentration is estimated, with approximately 95% confidence, by the interval

$$\log C = 1.20 \times \overline{\Delta_1/\Delta_2} - 1.01 \pm 0.16$$

where $\overline{\Delta_1/\Delta_2}$ is the mean of two determinations on the unknown solution. Figure 2 represents the regression of response Δ_1/Δ_2 versus the logarithm of concentration with approximately 95% confidence limits. This graph may be used as a nomogram of the mean Δ_1/Δ_2 is between 0.26 and 1.26. Thus, if the mean of two determinations of Δ_1/Δ_2 on a solution of MSH in Ringer's solution is 0.80, the concentration of MSH may be estimated to be between 0.39 and 0.82 unit per ml.

TABLE 2. ANALYSIS OF VARIANCE

Source of variation	Sum of squares	d.f.	Mean square	F	P
Regression	5.01	1	5.01	604	<0.0001
Deviation	0.04	3	0.0133	1.6	<0.05
Between	5.05	4			
Individuals	0.29	35	0.0083		
Total	5.34	39			

FACTORS INFLUENCING MELANOCYTE RESPONSE

Studies were carried out to determine the melanocyte response to variations in osmotic activity, ionic strength, pH, temperature, heavy metals, etc.

1. *Variations in the strength of Ringer's solution*

Frog skins were placed in a solution containing salts in the same proportion as Ringer's solution but of varying concentration. Light reflection was read at the time when the skin was transferred from the Ringer's to the test solution. The reading was taken after one hour. Then 1 unit of MSH per ml. of solutions was added, and the reflectance was read after one hour. The results are shown in Table 3. If the salt concentration was decreased below three-fourths of the concentration of Ringer's solution,

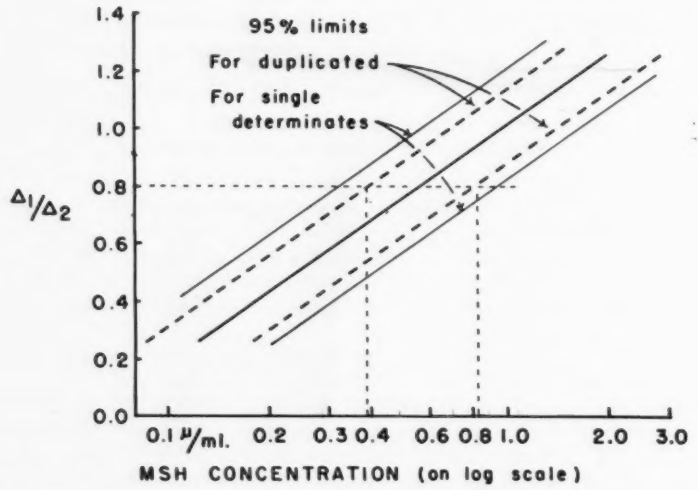

FIG. 2. Regression of response Δ_1/Δ_2 on logarithm of MSH concentration, with 95% confidence limits for single and duplicate determination.

TABLE 3. INFLUENCE OF MOLECULAR CONCENTRATION ON MSH EFFECT

Concentration of solution (Concentration of Ringer's solution is taken as 1.0)	Initial reading	After 1 hour	1 hour after MSH was added
3.0	98	100	98
	71	70	69
2.5	99	100	100
	77	72	69
2.0	92	95	98
	70	70	62
1.75	64	74	50
	86	87	84
1.5	83	81	61
	75	75	40
1.25	96	100	61
	66	70	34
1.00	77	74	42
	87	87	49
0.75	78	74	41
	78	75	38
0.50	74	62	52
	71	58	43
0.25	83	59	53
	74	58	57

the melanocytes darkened in color. If the salt concentration was increased to about 1.25× that of Ringer's solution, the effect of MSH decreased. The concentration of test solution should therefore be kept between 0.75–1.25× that of Ringer's solution.

2. *Variations in pH*

After the frog skin was stabilized in Ringer's solution, a reading was taken and the pH of the Ringer's solution was varied from 10.0 to 3.5. Sixty minutes later another reading was made. The results shown in Table 4 indicate that the pH of the solution must be kept above 6.5.

3. *Variations in temperature*

The influence of temperature was tested at 5° C, 17° C, 25° C., and 37° C. After the temperature of Ringer's solutions was stabilized, 1 unit per ml of MSH was added to the solution; and the reading was taken after 5, 10, 20, 30, 40, 50, 60, and 80 minutes. Then the skin was placed at room temperature, and 40 minutes later a new reading was made. The reading at this point was subtracted from the readings at other times, and the differ-

TABLE 4. INFLUENCE OF pH OF SOLUTION ON MSH EFFECT

pH	Reading of the skin before change of pH	Reading 1 hour later
10.0	69	69
9.3	82	80
9.0	79	79
7.0	75	76
6.5	85	83
6.5	66	67
6.0	63	51
5.7	74	69
5.2	83	64
5.0	67	52
4.8	66	55
4.5	90	73
4.0	65	36
4.0	65	36
3.5	90	52

ences were expressed as per cent of the difference between 0 and 120 minute readings. The results are shown in Figure 3. Each value is the average for several experiments. As shown in this figure, if the temperature were low, the effect of MSH was slow. At 37° C. melanocytes in Ringer's solution lighten more rapidly than at 25° C. However, darkening of melanocytes in

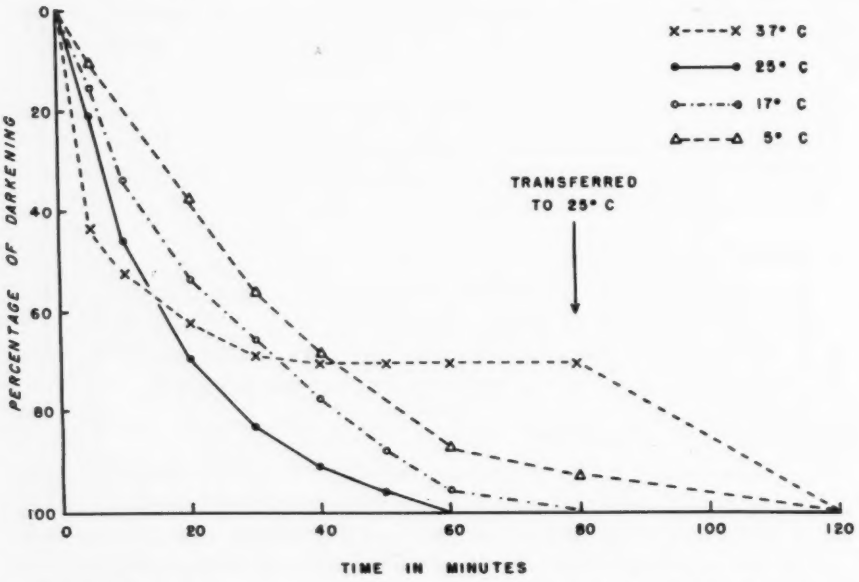

FIG. 3. Influence of temperature on MSH effect as determined by light reflectance.

the presence of MSH is less at 37° C. than at 25° C. The optimal temperature for this bioassay method seems to be between 20–25° C.

4. *Effect of heavy metals, sulfhydryl compounds, autonomic drugs, steroids, etc.*[4]

A reading was taken after the melanocytes in frog skin were allowed to lighten in 20 ml. of Ringer's solution. To one group 0.2 ml. of the solution of the substance to be tested was added. One hour later another reading was made, and the effect of the substance on the melanocytes was observed. To another group was added 0.2 ml. of the solution of the test substance with 20 units of MSH, and one hour later a reading was made. At least two frog skins were used for each experiment. Of the metals tested at a concentration of 1/100 M., manganese, cobalt, copper, iron, arsenic, zinc, aluminum, and mercury caused darkening of the melanocytes; but at a concentration of 1/1000 M., only mercury showed an appreciable effect. Two mg./ml. of sodium cyanide, 0.5 mg./ml. of ascorbic acid, 1.0 mg./ml. of sodium diethlydithiocarbamate, glutathione and cysteine; and 0.1 mg./ml. of acetylcholine, atropine, and histamine had neither a direct effect on melanocytes nor an inhibitory effect on MSH at the concentration test. This concentration is quite high for biological experiments. Pressor substances such as nor-adrenaline, adrenaline, hydroxytyramine, cobefrin, and sympathol had an inhibitory effect on MSH even at concentrations of 1.0 to 10.0 μg. per ml. Of the steroids tested, pregnane-3,11,20 trione caused darkening of melanocytes at concentration of 0.05 mg. per ml. Cortisone, hydrocortisone, testosterone, estradiol, 11-hydroxyprogesterone and 11-ketoprogesterone did not cause darkening of melanocytes or inhibit darkening by MSH even at concentrations of 0.1 mg. per ml.

5. *Effect of seasonal variation*

A very potent MSH preparation from the Armour Laboratory was tested for activity in January, April, and July. The results were as follows.

January	10.0 (6.9–14.5) $\times 10^8$ units per gm.
April	6.1 (4.2–8.8) $\times 10^8$ units per gm.
July	9.2 (6.4–13.2) $\times 10^8$ units per gm.

It can be seen that this method is fairly constant in different seasons.

When the MSH activity of an unknown solution is tested the latter should be neutralized and diluted to several concentrations, and then a small amount of each dilution (0.1 ml. or 0.2 ml.) should be added to the 20 ml. of Ringer's solution containing the frog skin so that changes of molar concentration and pH may be prevented. By testing these

[4] The details will be published later.

various dilutions the appropriate one having neither too strong nor too weak an effect on frog skin can be found. Final assay should be done using this dilution.

This method is rapid, twenty duplicate assays being performed easily within a day. Compared with Landgrebe and Waring's (1944) quantitative bioassay method, which requires 24 hypophysectomized toads for one preparation, this method is much more rapid and easier to carry out. It is not possible to compare the accuracy of our reflectance method with Frieden's absorption method because no statistical analysis was given by Frieden *et al.* (1948).

This method is especially useful for the isolation and identification of MSH, which requires a large number of assays. Possible interference by other substances can be eliminated easily by using dilution of 10^5 or greater.

SUMMARY

An *in vitro* quantitative bioassay method for melanocyte stimulating hormone (MSH) was described. This method depends upon the changes in light reflection from skin before and after immersion in solutions containing MSH. A photoelectric reflection meter is used for the measurements. Within certain limits the logarithm of MSH concentration is proportional to the ratio of the changes in reflectance obtained with unknown and the standard amounts of the hormone. Physical and chemical factors were shown to affect MSH activity. Optimum conditions consist of the use of Ringer's solution at 20–25° C.

Acknowledgment

We wish to express our thanks to Dr. Howard S. Mason for help given throughout this study, to Dr. Carl Hopkins for his aid in the statistical analysis and to Dr. Irby Bunding of the Armour Laboratories for the supply of beef posterior pituitary powder.

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A SIMPLIFIED TEST FOR ANTI-DIABETIC ACTIVITY

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IN A recent study of the quantitative relationships among glycosuria, food intake and body weight in alloxan-diabetic rats, an index of the severity of diabetes was established which was independent of changes in intake and body weight (Sturtevant, *et al.*, 1954). This index was obtained by dividing the glucose excretion by the amount of food consumed. The present report describes the effect of a known anorexigenic drug and of several dose-levels of insulin on this index.

MATERIALS AND METHODS

Forty-two male Sprague-Dawley rats, which varied from 100 to 300 gm. in weight, were given two successive intraperitoneal injections of alloxan monohydrate, 160 mg. per kg., 48 hours apart. After the 3 to 4 weeks required for stabilization of the diabetic glycosuria (Sturtevant, *et al.*, 1952), the rats were placed in individual metabolism cages and offered tap water and a medium carbohydrate diet (Table 1) *ad libitum*. The diet

TABLE 1. MEDIUM CARBOHYDRATE DIET*

Cellulose	120 gm.	Hawk & Oser salt mixture	40 gm.
Corn Starch	200	Wheat germ oil	10
Dextrin	190	Cod liver oil	10
Sucrose	200	Corn oil	200
Brewers' yeast	100		
Casein	160	Water	315 cc.

* Modified from Ingle *et al.*, 1946.

was given in the form of a thick paste to minimize spillage. The maximal available glucose and energy contents per gram of diet were calculated as 0.64 gm. and 4.8 Cal., respectively. Urine was collected daily, preserved with thymol and analyzed for glucose. Glycosuria was recorded daily for each rat in gm. glucose, food intake in gm. dry food, and body weight in gm.

After 5 days of control observations, the rats were divided into 5 groups and treated daily for a 5-day period as follows: group A (10 rats) received 32.0 U. per kg. of protamine zinc insulin (Lilly) subcutaneously, group B (9 rats) received 10.0 U. per kg., group C (8 rats) 3.2 U. per kg., and group D (9 rats) 1.0 U. per kg. Group E (6 rats) received 0.25 mg. per kg. of methyl *bis* (β-chlorethyl) amine hydrochloride (nitrogen mustard) intravenously.

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TABLE 2. INITIAL BODY WEIGHTS AND AVERAGES OF FOOD INTAKE AND GLYCOSURIA*

Group	No. rats	Drug	Dose, u. or mg./kg.	Pretreatment			Treatment		
				Weight, gm.	gm./rat/day		Weight, gm.	gm./rat/day	
					Intake	Glycosuria		Intake	Glycosuria
A	10	PZI	32.0	178	22.9	8.1	217	23.5	0.6
B	9	PZI	10.0	182	26.5	9.6	222	26.2	5.1
C	8	PZI	3.2	192	24.4	9.0	206	21.8	6.5
D	9	PZI	1.0	186	25.3	8.6	198	23.1	8.0
E	6	MBA	0.25	226	21.5	7.7	214	5.7	1.9

* G/I ratios calculated from individual rat ratios, not from these averages.
PZI = protamine zinc insulin.
MBA = methyl *bis* (β-chlorethyl) amine HCl.

RESULTS

The glycosuria and food intake of each rat were averaged over the last 4 days of the control period and over the last 4 days of the treatment period (Table 2). The ratios of the individual rat averages (glycosuria/intake, or G/I) were calculated and then averaged over all the rats of each group (Table 3). These mean ratios constituted the G/I indices of the severity of diabetes (Sturtevant, *et al.*, 1954).

TABLE 3. EFFECT OF TREATMENT ON THE G/I RATIO

Group*	G/I ratios ± S.E.		Difference, ΔG/I ± S.E.
	Pretreatment	Treatment	
A	.345 ± .012	.027 ± .005	.318 ± .013†
B	.360 ± .008	.189 ± .026	.171 ± .027†
C	.368 ± .009	.287 ± .027	.081 ± .028‡
D	.339 ± .012	.352 ± .013	−.013 ± .018
E	.363 ± .010	.346 ± .044	.017 ± .045

* See Table 2 for description.
S.E. = standard error.
† $P < 0.01$ that $\Delta G/I = 0$.
‡ $P < 0.05$ that $\Delta G/I = 0$.

Nitrogen mustard, which is known to be anorexigenic in alloxan-diabetic rats (Sturtevant, *et al.*, 1952), had no significant effect on the G/I ratio (Tables 2 and 3, Group E). On the other hand, increasing doses of insulin resulted in proportional decreases in the ratio (Table 3, Groups A–D). This relationship is illustrated in Figure 1 by a log dose-response curve, where the response is defined as the difference between the treatment and pretreatment G/I ratios for each group.

The regression illustrated in Figure 1 may be expressed in a slightly different manner. Since 64% of the diet is available glucose, G/0.64 I represents the proportion of available glucose which is excreted and 1.00 − G/0.64 I the proportion retained and presumably utilized. If these expressions are evaluated, using the ratios in Table 3, the differences between pretreatment and treatment values for each group represent the

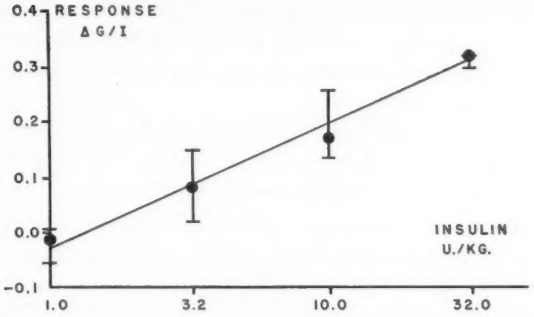

FIG. 1. Regression of the change of the G/I ratio on the $\log_{10}$ dose of protamine zinc insulin: $\Delta G/I = -0.028 + 0.222$ log dose insulin. Points are weighted by the reciprocals of their variances. The bars represent the 95% confidence limits. $P < 0.01$ that the true slope equal zero.

percentage decrease in excretion of available glucose or the percentage increase in its retention or utilization. These figures, which may be considered the observed improvement in glucose utilization, were calculated for the different groups: group A, 50%; group B, 27%; group C, 13%; group D, −2%; group E, 1%. Only the first three figures are statistically significant (Table 3).

In order to test the relationship of the G/I ratio with intake or body weight, correlation coefficients were calculated from the individual G/I ratios of each of the 36 rats receiving insulin. The G/I ratio was not significantly related to intake or body weight ($r = 0.04$ and 0.15, respectively).

The appropriateness of correcting for the regression of G on I by using a ratio instead of a linear expression* depends on the passage of the regression line approximately through the origin. On calculation, the y-intercept of the regression line was found to be close to zero (0, −1.3). The ratio method of correction thus appeared to be validated.

DISCUSSION

The glycosuria of diabetic animals can be corrected for the effect of food intake in several ways, but most are subject to criticism. One common practice is to force-feed the animals. This procedure can produce alimentary glycosuria by over-feeding (Ingle, 1946), although Ingle's force-fed diabetic rats are adapted to the procedure and are commonly under-fed (Ingle, 1947). Force-feeding is a wholly unnatural form of alimentation in the rat, which characteristically eats continually and not at single large meals.

Another common practice employs pair-fed controls. These animals may

* $G - bI$, where b is the slope.

eat less, but not more, than the amount of food offered, while the treated animals may eat as much or as little as they desire. The variability of the control data is intentionally restricted and the interpretation of the experimental results becomes difficult.

The use of the G/I ratio avoids these criticisms and has several advantages. Each group of rats is used as its own control, so that the results may be calculated on a paired-observation basis, if desired. The animals are allowed to continue in as "natural" a state as possible. Random variations in glycosuria, food intake, and body weight are allowed to occur in both the control and treatment periods. Most important, the anorexigenic and the anti-diabetic properties of a drug may be separated. The G/I ratio is not related to intake (or body weight), but to the capacity of the animals to retain and presumably utilize the glucose consumed.

For the present, the G/I ratio may be regarded as a tentative index of insulin deficiency. The effect on the ratio of other factors in diabetes (e.g. glucocorticoids, thyroxin, folliculoids) is presently under investigation.

SUMMARY

An index of the severity of alloxan diabetes in rats has been established as the percentage of food consumed which is excreted as glucose. This index is not related to food intake or body weight. It decreases linearly with increasing log doses of insulin and is unaffected by anorexigenic doses of nitrogen mustard.

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THE EFFECT OF GOITROGEN WITHDRAWAL ON THE PITUITARY-THYROID SYSTEM OF THE GUINEA PIG[1]

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THERE have been relatively few studies on the rehabilitation of the pituitary-thyroid system following the extensive changes induced in these glands by the administration of anti-thyroid agents. Morphologic alterations are known to persist in the thyroid of various species long after goitrogen withdrawal (Astwood, Sullivan, Bissell, and Tyslowitz, 1943; Mackenzie and Mackenzie, 1944; Engle and Aranov, 1946; Webster, 1949) but the status of thyroid and pituitary function under these conditions has received scant attention. There are certain indications that restoration of the normal pituitary-thyroid balance after thyroid inhibition may be characterized by an initial period of temporarily enhanced function. D'Angelo, Paschkis, Cantarow, Siegel, and Rivero Fontan (1951) have demonstrated in guinea pigs treated for prolonged periods with propylthiouracil (PTU), that depressed thryoid uptakes of I^{131} quickly rebound above the normal range after goitrogen withdrawal and that increased radioactivity in such glands reflects not only increased tissue mass but also augmented avidity for radioiodine ("rebound phenomenon"). In comparable studies on the rat, the return of thyroid uptake of radioiodine to normal following release from PTU is long delayed but the rebound phenomenon has been observed in acute experiments (D'Angelo, Paschkis, Cantarow, Siegel and Friedler, 1952). The time relationships involved in this phenomenon of augmented iodine fixation and the extent to which the hypophysis and other phases of

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thyroid activity are affected by goitrogen withdrawal remain to be clarified. In the present study, the rebound phenomenon of thyroidal radioiodine has been re-examined in the guinea pig with two major objectives in view; namely, 1) to determine its duration, and 2) to elucidate its significance as regards overall thyroid function. Thyroid-pituitary interplay has been evaluated during and after PTU treatment by measurement of the following parameters: I^{131} uptake and distribution in the thyroid, serum protein bound iodine (PBI), serum thyrotrophic hormone (TSH), oxygen consumption (BMR), and thyroid histology. The effect of goitrogen withdrawal has been studied in animals over a period of 1–40 days following continuous treatment with PTU (60–200 days). A preliminary account of the results has been published (Paschkis, D'Angelo, Cantarow, Sunderman and Friedler, 1953).

MATERIALS AND METHODS

Approximately 200 guinea pigs were used in the various experiments. Young adult female guinea pigs (350–400 gm. body weight), procured locally, were maintained in the laboratory on unrestricted amounts of Purina Omolene chow (iodine content—0.0001%) and tap water. This diet was supplemented daily with ample amounts of fresh greens. PTU was administered in the basic ration (0.1%). Animals were maintained on this regime for periods ranging from 2–200 days.[7] Except for absence of the drug in the basic ration, treatment of control animals was identical. The effects of withdrawal of the goitrogenic agent were investigated during a period of 1–40 days following 60–200 days of uninterrupted treatment.

For the radioiodine studies, guinea pigs were injected intraperitoneally with a tracer dose of I^{131} (8 μc., without carrier)[8] 24 hours prior to sacrifice. Single thyroid lobes were homogenized in 2% NaOH. The thyroid suspension (0.5 ml.) was pipetted onto planchettes and, after drying, their radioactivity was measured and compared with aliquots of the original sample injected. The proportions of radioiodine in the inorganic ("Non-precipitable") and organically-bound iodine fractions of single thyroid lobes were determined after trituration in saline solution and precipitation with 10% trichloroacetic acid. Oxygen consumption was measured by a modification of the technic of Grad (1952). Animals were placed in individual chambers and oxygen consumption was determined after a 30 minute acclimatization period in a single 10–15 minute run.[9] Simultaneous determinations were made once daily on experimental and control animals. (The coefficient of variation in oxygen consumption of the normal female guinea pig, measured over 6 successive days, averaged 14.2%.) Blood was withdrawn from lightly anesthetized animals by cardiac puncture and without anticoagulant. All blood determinations were made on pooled sera. Serum protein bound iodine was measured in duplicate by a modification of the ceric-arsenite reduction method of Barker, Humphrey and Soley

[7] Growth of the female guinea pig (like that of the male; Brown and Young, 1952) is essentially normal throughout 80–100 days of PTU treatment, but there was some indication of reduced growth in the present study after 4–5 months of goitrogen administration. Mortality was also appreciable with prolonged treatment.

[8] I^{131} was obtained from the Atomic Energy Commission, Oak Ridge.

[9] Grateful acknowledgement is made to Miss Rose Nakayama for technical assistance.

(1951). Serum TSH was ascertained by the stasis tadpole technic (D'Angelo and Gordon, 1950). The thyroids and hypophyses were weighed routinely on a micro-torsion balance at autopsy. Thyroid histology was studied on Bouin fixed, hematoxylin and eosin, or Masson stained paraffin sections (5 micra).

RESULTS

The effect of prolonged administration of PTU on thyroid and pituitary weight is shown in Table 1. The major morphologic changes occurring in these glands were found to be similar to those described previously in experiments of shorter duration (D'Angelo *et al.*, 1951; D'Angelo, 1953). Approximately 2 weeks of PTU treatment were required to induce hyper-

TABLE 1. PARAMETERS OF PITUITARY-THYROID FUNCTION IN THE GOITROUS GUINEA PIG

Mean body wgt. gm.	No. pigs	Days on drug	Mean pit. wgt., mg./100 gm. body wgt.	Mean thy. wgt., mg./100 gm. body wgt.	Mean thyroid I^{131} uptake (%)		PBI μg. per 100 ml.	Blood thyroid hormone —TSH balance	
					100 gm.	Gland		Hindlimb increase (mm.)	Thyroid cell hgt. (μ)
556±81[1]	52	0	2.3±0.13	14.4± 1.5	4.4± .7	3.2± .2	2.4± .2[2]	2.3 ±0.3[2]	5.3±0.1
352±29	9	2–8	2.5±0.25	16.1± 0.9	.7± .1	.4± .1	1.5	1.9±0.2	5.8±0.1
324±42	5	15–18	2.5±0.20	14.8± 2.9	1.2± .2	.7± .2	—	1.5±0.1	5.8±0.1
348±24	6	23–29	2.5±0.20	24.5± 2.8	1.0± .1	1.2± .1	.5	2.0±0.1	6.2±0.1
523±52	3	47	2.6±0.20	45.5±12.4	.6± .3	1.4± .4	.3	1.5±0.1	6.2±0.1
586±3	3	60	2.6±0.10	41.3± 6.5	2.0± .2	5.3± .7	.5	1.3±0.2	6.2±0.1
525±111	5	80	2.7±0.18	76.7±11.2	.9± .1	3.3± .4	.5	1.2±0.1	6.2±0.1
520±61	3	100	3.2±0.23	149.0±44.4	.7± .1	5.7±1.2	1.2	.9±0.1	6.4±0.1
518±60	4	200	3.3±0.20	139.1±20.4	.9± .1	6.2±1.3	1.0	1.6±0.2	6.2±0.1

[1] Mean wgt. ± average deviation; in all other columns ± values represent standard error of the mean.
[2] Represents a grand mean value based on determinations in 8 separate groups (Range, 1.8–3.5 μg. per 100 ml.).
[3] Refers to mean hindlimb increase and mean thyroid cell heights in test tadpoles (Rana pipiens or clamitans) receiving 0.05 ml. of serum (once daily, 6 days). Cell heights of 5.8 micra and 6.4 micra are equivalent to 0.3 μg. and 0.6 μg. TSH respectively of a standardized preparation per milliliter of serum. No TSH was detected in normal guinea pig serum.

trophic changes in the thyroid epithelium. The thyroid enlarged after 3–4 weeks of treatment. Thyroid hyperplasia thereafter was progressive, with maximal enlargement occurring after 100–200 days of PTU administration. Pituitary hypertrophy eventually ensued (80 days) and after 100–200 days of treatment the increase in hypophyseal weight was approximately 50%. The enlargement of the pituitary could be accounted for primarily by hypertrophic changes in the adenohypophysis.

The depression in thyroid uptake of I^{131} with PTU was prompt and marked, and was maintained during 200 days of uninterrupted treatment (Table 1). The decrease in I^{131} uptake per milligram of wet thyroid tissue was of the same order of magnitude throughout, but total radioactivity in markedly hyperplastic glands (100–200 days) exceeded normal. The changes found in the thyroid hormone-TSH balance and in the PBI of serum correlated closely with the reduction in thyroid uptake of radioiodine (Table 1). The pattern of changes in these parameters of thyroid activity signified inhibition of thyroid hormone formation and release during administration of PTU. Serum PBI levels were significantly lowered and

blood titers of TSH were elevated at all time intervals studied. The rise in blood TSH occurred before an appreciable anatomical change was noticeable in the thyroid. TSH concentration in the blood increased to slightly higher levels when the thyroid began to enlarge. TSH blood levels thereafter remained relatively constant despite the progressive hyperplasia of the gland. The lesser increase in hindlimb extension which occurred in test tadpoles receiving serum relatively high in TSH content was indicative of a relative deficiency in the thyroid hormone activity of these sera. This diminution in metamorphosing capacity of sera paralleled the reduction in the PBI.

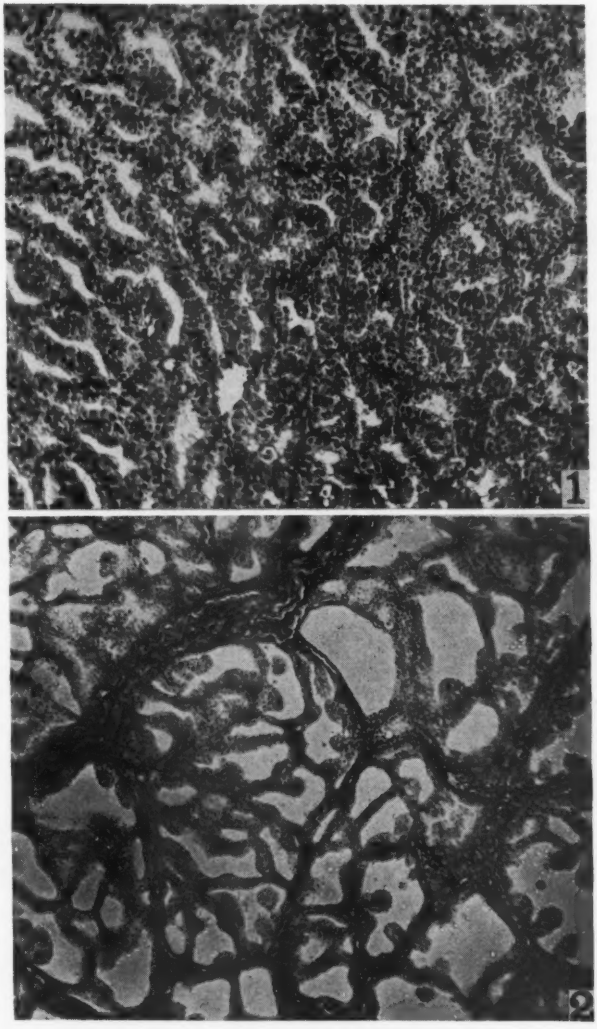
Maximal histologic change in the thyroid with PTU was achieved after 80–100 days of continuous treatment. The gland was depleted of colloid or

TABLE 2. EFFECT OF GOITROGEN WITHDRAWAL ON THYROID FUNCTION IN THE GUINEA PIG

Days on drug	No. pigs	Mean thyroid (mg.) 100 gm. body	Mean thyroid I^{131} uptake %		Mean B.M.R. cc. O_2/hr./100 g.	P.B.I. (μg. per 100 ml.)	Serum TSH mean thyroid cell hgt. (μ)
			100 mg.	Gland			
0	8	15.9	4.0±0.7*	3.3±0.5	88.4±2.9	2.2	5.0±0.1
60	3	41.3	2.0±0.2	5.3±0.7		0.5	
—Off 1	1	70.0	4.0	12.7		5.5	
—Off 2	5	58.7	6.7± .9	21.5±3.1		0.7	
—Off 18	3	20.6	3.7±0.4	4.0±0.4		2.9	
80	5	76.7	.9±0.1	3.3±0.4	78.1±2.9	0.5	6.2±0.1
—Off 1	1	79.8	4.0	18.4		2.0	
—Off 5	2	49.0	7.8	21.8	76.7±0.5	1.8	5.9±0.1
—Off 9	5	41.8	8.0±1.0	16.3±5.1	86.7±3.9	2.4	
—Off 15	9	46.6	15.0±3.3	24.9±5.8	88.3±5.0	1.8	5.7±0.1
—Off 32	3	37.6	6.0±1.1	11.6±3.3	85.0±2.3	1.9	
100–160	4	151.5	.9±0.1	7.2±1.5		0.8	6.3±0.1
—Off 5	5	113.4	7.0±1.4	47.5±6.8		5.4	6.0±0.2
—Off 15	3	75.0	9.0±1.5	35.3±8.5		2.0	5.8±0.2
—Off 40	4	69.7	2.0±0.0	8.6±1.2		2.3	5.3±0.1

* In all columns, ± values represent standard error of mean.

contained vestiges of feebly staining material, usually in peripheral follicles. The vascularity was high throughout and the central portion of the gland consisted largely of solid epithelial masses in which the original folliculation was lost. The tall columnar epithelium contained either large, ovid nuclei or pycnotic, irregularly-shaped ones. Mitoses were evident. There was thickening of the thyroid capsule and stroma. No neoplastic changes were seen. Withdrawal of PTU after 80–160 days of administration resulted in slow regression of the hyperplastic thyroid (Table 2). Pituitary weights were also decreased. The thyroid was still enlarged 30–40 days after release from PTU. Withdrawal of the goitrogen drastically altered the histologic picture. The changes undergone by the thyroid in involution are illustrated in Figures 1-6. The major histologic changes were not evident until 2–5 days after withdrawal of PTU (cf. Figs. 2 and 4). At this interval, abundant amounts of deeply-staining homogeneous colloid reappeared in the gland. Formation of colloid restored in some measure



Figures 1–6 are unretouched photomicrographs of sections illustrating representative areas in the central portion of the thyroid (Bouin's picroformol; hematoxylin and eosin).

FIG. 1. The degree of morphologic change after 100 days of PTU treatment is depicted. Colloid depletion is practically complete. ×100.

FIG. 2. The considerable re-deposition of colloid, as well as stromal changes, is shown after 5 days of goitrogen withdrawal (PTU-100 days). ×100.

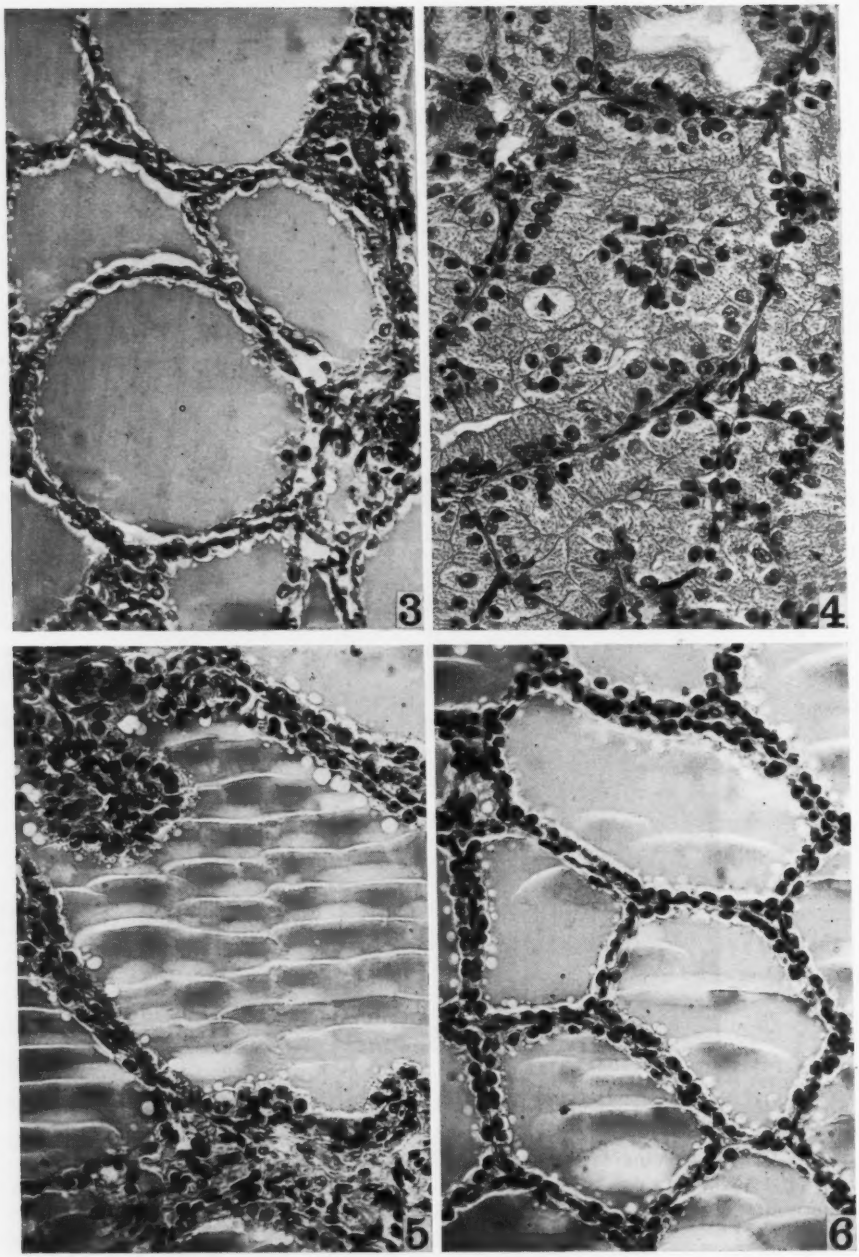

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TABLE 3. DISTRIBUTION OF I^{131} IN THE THYROID DURING AND AFTER PTU TREATMENT

Days on drug	No. pigs	Mean thyroid wgt. (mg.)	Mean P.B.I. μg. per 100 ml.	Mean thyroid I^{131} uptake —% inj. dose			
				per 100 mg.	as organic	as inorganic	total gland
0	4	59.6	1.8	4.88	4.80	.08	2.9
100	3	308.5	1.1	1.81	.67	1.13	5.5
140; Off 40	4	406.8	2.3	2.10	2.00	.05	8.5
0	6	94.7	2.2	4.20	4.00	.10	3.9
200	3	643.0	1.2	.90	.04	.05	5.8
200; Off 5	4	757.5	2.4	.64	.60	.04	4.5
0	4	105.1	3.2	2.63	2.60	.03	2.8

the original folliculation, but the general morphologic pattern was bizarre. The lumina were highly irregular in shape and entrapped large islands of epithelial cells containing extravasated hemal elements. The thickened fibrous connective tissue traversed the gland, producing a lobulated appearance. As involution progressed, more colloid appeared and the follicular epithelium was lowered in height (Figure 5). The colloid in these still enlarged glands was dense and hardened and showed minimal vacuolation. Some distended follicles were unusually large. Thirty two–40 days after goitrogen withdrawal the gland approached normal histologic appearance (Figs. 3 and 6). Follicular size and shape were normal but the acinar epithelium still remained slightly elevated.

A vigorous rebound of the thyroid uptake of radioiodine to supernormal levels followed withdrawal of PTU after 60–160 days of treatment (Table 2; Figure 7). The rise in I^{131} uptake (per milligram of wet tissue) was evident as early as 24 hours after release from PTU. Within 2–5 days after goitrogen withdrawal, the uptake of I^{131} by the thyroid was approximately twice normal. The increase in total radioiodine collection by the gland 5 days after goitrogen withdrawal was approximately 600% and 1400%, respectively, for the 80 day and 100–160 day intervals of PTU treatment. Peak values for the augmentation in radioiodine uptake per milligram of

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FIG. 3. The normal gland is shown with low epithelium and distended follicles, characteristic of the "resting" state. ×312.

FIG. 4. The hypertrophic changes in the follicular epithelium and the obliterated lumina are shown 1 day after goitrogen withdrawal (PTU-60 days). ×312.

FIG. 5. A portion of a large follicle in the regressing thyroid is indicated. The dense, hardened colloid has entrapped cellular masses. The follicular epithelium is still elevated and vascularity is high (PTU-80 days; off 15). ×312.

FIG. 6. The section illustrates the degree of thyroid involution 32 days after PTU release (on drug 80 days). Some morphologic change persists but the gland approaches the normal histologic condition. ×312.

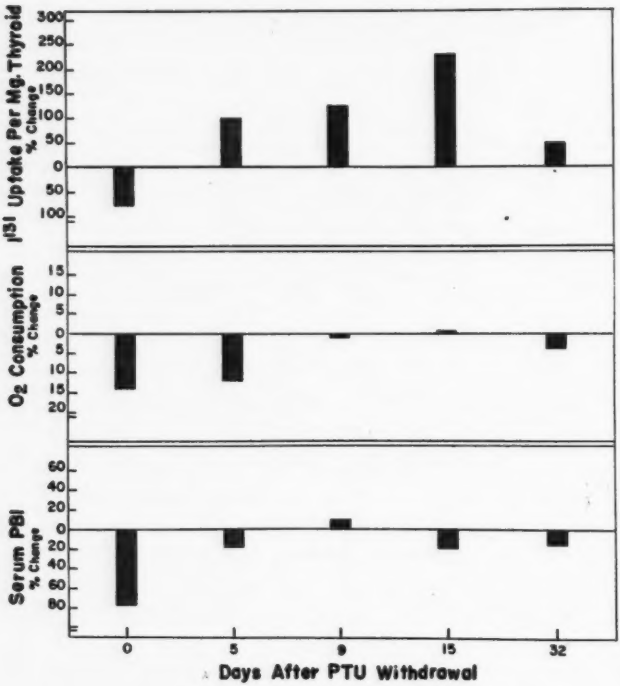

FIG. 7. The pattern of change in thyroidal radioiodine uptake, oxygen consumption, and serum PBI is shown after release from 80 days of continuous PTU treatment. The values are expressed as percent change of normal, with bars above and below 0 line representing increase and decrease respectively.

thyroid tissue were achieved 15 days following goitrogen withdrawal. The decrease in thyroid mass at this time, however, was sufficient to offset the increased avidity, and total radioactivity in the gland began to fall. The rebound effect had subsided by 18–40 days after PTU release, although total radioiodine accumulation in the still enlarged glands exceeded normal. A rebound in I^{131} uptake failed to occur after PTU release in animals receiving the goitrogen for 200 days. The uptake 5 days after goitrogen withdrawal was still considerably below normal. Inasmuch as only one time interval of withdrawal was studied here, it cannot be stated whether the time sequence of the effect was changed or whether the phenomenon could no longer be elicited.

The increase in iodine-collecting activity of the thyroid which resulted from goitrogen withdrawal was not accompained by commensurate change in other indices of thyroid function (Table 2; Figure 7). The decrease in

BMR induced by 80 days of PTU treatment was 11.6% of normal. PTU withdrawal restored oxygen consumption to normal after an appreciable time lag, but no indication of further augmentation of oxygen consumption was found. No apparent correlation existed between the course of the thyroid uptake of radioiodine and the BMR; the former rose and fell with oxygen consumption at either reduced or normal levels. The pattern of change in the level of serum PBI was similar to that in the BMR. Serum PBI rose to approximately normal levels after goitrogen withdrawal. Rebound of the serum PBI to excessively high levels did occur in two instances (PTU 60 days, 1 day off; PTU 100–160 days, 5 days off). These sera also possessed increased metamorphosing capacity when tested in tadpoles. The elevation of PBI was not sustained however, whereas the augmentation in I^{131} uptake was. It is possible that supernormal levels of PBI may have occurred more frequently after goitrogen withdrawal but were missed because of their brief duration. Although changes in oxygen consumption and serum PBI could not be correlated with the rebound in thyroid uptake of radioiodine, it appeared that the occurrence of the latter was contingent upon excess TSH in the circulation (Table 2). TSH blood levels were elevated in all cases in which the uptake of I^{131} per mg. thyroid tissue was greater than normal. High TSH titers, however, did not necessarily portend a rebound effect (e.g., 200 days PTU, 5 days off) and, in fact, levels of TSH in blood declined progressively after goitrogen with-

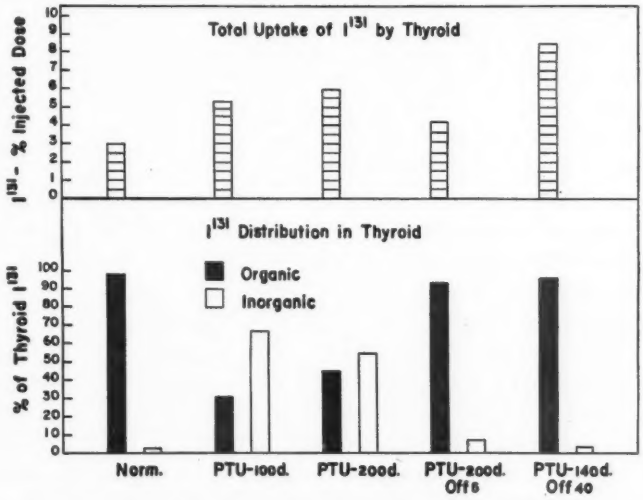

FIG. 8. The upper graph (horizontal lines) indicates the total I^{131} collection by the thyroid gland. The height of the dark and light bars represents the percentage of the total radioiodine found in the organic and inorganic fractions of the gland respectively.

drawal. The changes in blood TSH thus did not parallel the rise and fall in the I^{131}-accumulating activity of the thyroid.

The thyroids of guinea pigs maintained on PTU for 100 and 200 days were characterized by a substantial change in the proportions of inorganic and organically-bound radioiodine (Table 3). The distribution of the administered I^{131} between these two fractions is presented in Figure 8. In normal glands the inorganic and organically-bound iodine fractions averaged 2.7% and 97.3%, respectively, of the total radioactivity in the gland. In the thyroids of animals kept on PTU for 100-200 days, organically-bound radioiodine values were reduced approximately $\frac{1}{2}$ to $\frac{1}{3}$, with 50–70% of the labeled iodine recoverable in the inorganic fraction. Five days after goitrogen withdrawal, the distribution of I^{131} in both iodine fractions was restored to nearly normal proportions. This occurred despite the fact that I^{131} uptake per mg. of thyroid in this experiment (200 days PTU, off 5 days) was still markedly depressed (Table 2). (Fractionation of the thyroid was not done in the rebound series of experiments.) The labeled iodine moieties of the thyroid eventually attained normal proportions after release from PTU inhibition (PTU 140 days, 40 days off).

DISCUSSION

These observations make it possible to analyze more closely than heretofore the mechanism of pituitary-thyroid interplay in the guinea pig during and after prolonged administration of an antithryoid agent. As in other species, the reduction in thyroid uptake of radioiodine induced by PTU is prompt and marked. The sequential reactions to thyroid hormone formation are altered, as indicated by a shift in the distribution of labeled iodine in the gland fractions. The total fixation of radioiodine in the goitrous gland may exceed normal but, as in the rat (Astwood, 1944–45; McGinty and Sharp, 1946; Taurog, Chaikoff and Feller, 1947; and VanderLaan and VanderLaan, 1947), a large portion of its fails to become organically bound. The observation of increased blood TSH titers with concomitantly reduced levels of serum PBI is consistent with the concept that thyroid hyperplasia results from excessive pituitary TSH secretion consequent to inhibited thyroid hormone formation (Mackenzie and Mackenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943). With prolonged PTU treatment, TSH concentration in the guinea pig's adenohypophysis rises and the augmented production of the hormone is clearly associated with an increase in the size, number, and cytologic activity of the basophilic cells (D'Angelo, 1953). TSH production, normally at a low level, apparently must increase to insure adequate delivery of the hormone into the circulation. A similar situation exists for the cold-exposed guinea pig (Stevens, D'Angelo, Paschkis, Cantarow and Sunderman, 1953). The constancy of

blood TSH levels throughout prolonged PTU treatment indicates that thyroid hyperplasia is a cumulative response to a sustained effective concentration of circulating TSH.

The response of the pituitary-thyroid system of the guinea pig to PTU differs from that of the rat in several respects. These differences are referable primarily to adjustments in the homeostatic mechanism regulating pituitary-thyroid interaction rather than in the fundamental nature of the interplay. TSH content of the normal rat pituitary is relatively high. PTU treatment substantially reduces it and blood TSH levels rise correspondingly (D'Angelo, Stevens, Paschkis and Cantarow, 1953). In contrast to the rat, there is an appreciable lag in the appearance of morphologic change in the guinea pig thyroid with PTU (D'Angelo *et al.* 1951, 1953; Brown and Young, 1952). A relatively slow rate of peripheral utilization of circulating thyroid hormone (with consequent lag in pituitary TSH response) may be a factor in this delayed goitrogenesis (Rand, Lee and Williams, 1951) but cannot account for it completely; blood TSH levels in the present study were increased long before thyroid enlargement occurred. The inherent sensitivity of the thyroid to TSH must also be considered. The relatively slight decrease in oxygen consumption found in the goitrous guinea pig confirms observations made by Peterson, Webster, Rayner and Young (1952). The fall in BMR in the goitrogen-treated rat is much more pronounced (Mackenzie and Mackenzie, 1943; Astwood *et al.*, 1943; Barker, Kiely and Lipner, 1949, and others). The degree of hypothyroidism induced in the guinea pig either by anti-thyroid agents or by thyroidectomy (Young, Rayner, Peterson and Brown, 1952) is relatively mild. The slight decrease in metabolic rate, the inactive histologic picture of the thyroid, its low level of radioiodine uptake, the low concentrations of TSH in blood and pituitary—all suggest that under ordinary laboratory conditions the thyroid-pituitary system of the guinea pig functions at a relatively low level.

The necessity for simultaneous measurement of as many parameters as possible in studies of the pituitary-thyroid interrelationship is clearly shown. The present work has demonstrated that a relatively long sustained augmentation of the iodine collecting function of the thyroid[10] can be induced by withdrawal of the chemical agent initially inhibiting it—a phenomenon apparently unaccompanied by equivalent change in other indices of thyroid function. Although the rate of release of thyroid hormone from

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[10] The possibility has not been precluded that the increased thyroidal I^{131} uptake after PTU withdrawal results, at least in part, from extra-thyroidal changes (e.g., alteration in iodide space or in renal excretion). The rebound in I^{131} uptake, however, is high and likely reflects substantial increase in I^{127} uptake by the thyroid as well.

the gland was not measured, there was little to suggest that this phase of thyroid activity waxed and waned as did the radioiodine uptake. High serum PBI levels after PTU withdrawal were found in only a few instances, and the gradual return of oxygen consumption to normal without rebound did not point to unduly high peripheral utilization of thyroid hormone. It thus appears that rehabilitation of the thyroid gland following release from PTU block may be characterized by a dichotomy of function. This dissociation of thyroid activities permits hyperactivity of the iodine-fixing mechanism of the gland without the induction of a hyperthyroid condition in the organism.

A satisfactory explanation for the rebound phenomenon of I^{131} uptake by the guinea pig thyroid cannot be given at present. It has been speculated (D'Angelo *et al.*, 1951) that prolonged administration of PTU may enhance the activity of an iodide-concentrating mechanism in the thyroid (in response to endogenous TSH). Upon withdrawal of the goitrogen, the block to organic binding is rapidly removed and the TSH-activated mechanism now permits iodine uptake and formation of thyroid hormone to proceed at a high rate. We have obtained some evidence (unpublished experiments) that the ratio of thyroid iodide to serum iodide in the guinea pig is markedly increased with PTU treatment. The distribution of labeled iodine in the thyroid reveals, moreover, that the ability of the gland to bind iodine organically is soon restored after goitrogen withdrawal—an event which coincides with colloid accumulation. The failure of increased release of thyroid hormone to occur from these hyperplastic thyroids despite high titers of circulating TSH, represents a significant difference from the condition in glands activated with exogenous TSH. Morton, Perlman and Chaikoff (1941) have shown that in the guinea pig thyroid activated with exogenous TSH, radioiodine uptake is increased, and that the gland contains more organically bound radioiodine than normal, with an increased proportion of thyroxine. In such glands there is colloid release, and both production and release of thyroid hormone are accelerated. In the hyperplastic thyroid after goitrogen withdrawal, there is increased radioiodine uptake with marked colloid accumulation, but release of thyroid hormone is apparently normal.

Two possible explanations may be offered as to why the release phase of thyroid activity fails to parallel the augmentation in the iodine-seizing capacity of the gland. It is conceivable that, even though chemical pathways to thyroid hormone formation are re-established after goitrogen withdrawal, the amount of thyroid hormone formed is just sufficient to account for normal delivery to the circulation. This would imply that large amounts of inorganic iodine present in the hyperplastic gland inhibit appreciably the organic binding of iodine and, consequently, the formation of thyroid

hormone. That such a mechanism may exist in the rat thyroid has been demonstrated both *in vitro* (Morton, Chaikoff and Rosenfeld, 1944) and *in vivo* by Wolff and Chaikoff, (1948). It should be emphasized however, that this inhibitory action of excess iodide on organic binding in the thyroid is temporary in nature (Wolff, Chaikoff, Goldberg and Meier, 1949). It is equally possible, on the other hand, that the hyperplastic thyroid of the guinea pig after PTU release manufactures large amounts of thyroid hormone, but that some mechanism for its release has been disturbed. Some evidence has been presented that the mobilization and release of thyroglobulin from the follicle involves participation of a proteolytic enzyme system which is activated by TSH and suppressed by excess iodide (DeRobertis, 1941; Dziemian, 1943; DeRobertis and Nowinski, 1946). Accordingly, the great influx of iodine into the thyroid after goitrogen withdrawal may interfere with the response of a proteolytic mechanism to circulating TSH so that thyroid hormone is not released at a rate commensurable with its formation.

Whatever may be the explanation for the dichotomy in thyroid function, it would appear that prolonged administration of PTU may differentially alter two mechanisms in the thyroid, one leading to thyroid hormone formation, the other to its release. The significance of the change which occurs in these thyroid mechanisms after goitrogen withdrawal will perhaps become more readily apparent when hormonal and non-hormonal iodine in the thyroid are determined quantitatively, and when the effects of hypophysectomy and exogenous TSH are investigated.

SUMMARY AND CONCLUSIONS

The functional status of the pituitary-thyroid system in the guinea pig has been investigated during and after prolonged treatment with PTU. Administration of the goitrogen (2–200 days) produced the following results:

1) Thyroid hyperplasia and eventual enlargement of the pituitary.

2) Prompt and sustained reduction in thyroid uptake of I^{131}.

3) Alteration in the distribution of labeled iodine in the inorganic and organic fractions of the thyroid, which were increased and decreased, respectively.

4) Elevation of serum TSH, which was maintained with relatively little fluctuation.

5) Diminution in serum PBI levels and in oxygen consumption.

Rehabilitation of the pituitary-thyroid axis was studied 1–40 days following withdrawal of the goitrogen. Within 2-5 days after release from PTU, the slowly regressing thyroid acquired considerable amounts of colloid. The gland was still enlarged 40 days after goitrogen withdrawal.

Pituitary weights decreased to normal. The I^{131} inorganic and organic fractions were restored to normal proportions. There was a rebound of depressed thyroid uptake of radioiodine (per mg.) to supernormal levels (200–300%), which persisted for at least 15 days after release from PTU block. BMR and serum PBI returned to normal without rebound. Serum TSH titers declined progressively.

The observations indicate that the administration of PTU may differentially alter two mechanisms in the guinea pig thyroid, one leading to formation of thyroid hormone, the other to its release. Goitrogen withdrawal may temporarily establish a dichotomy in thyroid gland function. This dissociation of thyroid activities results in hyperactivity of the iodine-seizing mechanism of the gland without inducing a hyperthyroid state in the organism.

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OBSERVATIONS ON *IN VITRO* ADRENAL STEROID SYNTHESIS IN THE RAT[1]

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INTRODUCTION

THE *in vitro* production of adreno-cortical steroids has been demonstrated in the rat by Saffran *et al.* (1952), in the cow by Haynes *et al.* (1952b) and in the dog by Brady (1953). Both Saffran *et al.* and Haynes *et al.* were also able to demonstrate an *in vitro* stimulatory effect of ACTH on corticoid output.

In the work about to be reported, we have endeavored to evaluate the effects of various compounds on the *in vitro* formation of steroids by rat adrenal glands.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain, weighing about 150 gm., were used in the experiments described below.

The incubation procedure was as follows: the animals were sacrificed by manual separation of the cervical vertebrae and the adrenal glands obtained as rapidly thereafter as possible. The glands were cleaned of adherent tissues, weighed on a Roller-Smith torsion balance and cut into quarters with a razor blade. Our incubation vessels were 25 ml. Erlenmeyer flasks, each flask receiving the quartered glands from two animals. In addition, each flask contained 3 ml. of our incubation medium (0.124 *M* NaCl, 0.0142 *M* K_2HPO_4, 0.0058 *M* KH_2PO_4 and 0.011 *M* glucose). The pH of the medium was 7.28. The final molar concentrations of the various substrates used are described below. The flasks were then gassed with 100% oxygen for 5 minutes, stoppered and incubated, with gentle shaking, for $2\frac{1}{2}$ hours at 37° C. Following the incubation period, they were stored at −20° C. until analyzed. Routinely, twenty flasks were incubated in each experiment.

The general analytical scheme was that of Haynes, Savard and Dorfman (1952a). For the purposes of extracting and purifying the steroid fractions in our incubation mixtures, 6 ml. of reagent grade ethyl acetate (Meyer, 1953) were first added to each flask and extraction effected by swirling them fifty times. The ethyl acetate was poured off

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into another flask and this procedure repeated two additional times. The combined ethyl acetate extracts were then washed once with 3 ml. of water, and the organic phase quantitatively transferred to 300 ml. round bottom flasks. The ethyl acetate was then distilled off in an all glass apparatus under a water-pump vacuum, the temperature in the water bath being maintained between 60° and 65° C.

The residue in the round bottom flasks was taken up in a total of 3 ml. of benzene and put on a silica gel column for chromatographic purposes (Levy *et al.*, 1953). The column consisted of a 50 ml. reservoir connected to a 10 cm. length of 6 mm. glass tubing and thence to a stopcock. Into the tube a slurry of 0.5 gm. of silica gel (Davison Chemical Co., Baltimore, Md., 100–200 mesh) in benzene was placed. The column of gel was supported by a small plug of glass wool. The benzene extract was run through the column, and then the column was eluted by five successive solvents or solvent mixtures:

Fraction I	16 ml. of benzene
Fraction II	16 ml. of benzene-ethyl acetate (5:1 v/v)
Fraction III	16 ml. of benzene-ethyl acetate (4:1 v/v)
Fraction IV	16 ml. of ethyl acetate
Fraction V	16 ml. of methanol.

Routinely, only the first four fractions were obtained. The first three were generally discarded, only Fraction IV being analyzed for its content of formaldehydogenic compounds. As subsequent observations demonstrated that Fractions I and II could be safely omitted, in the later phases of our work the columns were eluted only with 10 ml. of Fraction III and 16 ml. of Fraction IV. Fraction V was only obtained on special occasions, as will be described later.

Once obtained, Fraction IV was taken to dryness in a water bath at 50° to 55° C. under a stream of nitrogen or carbon dioxide. To the dry sample were then added 0.2 ml. of glacial acetic acid (Baker's Special Analyzed), 2.0 ml. of water and 0.1 ml. of 0.05 *M* periodic acid in 0.3 N sulfuric acid in that order. This procedure will effect the formation of formaldehyde only in those compounds containing a terminal carbon which has an amino or a primary hydroxyl group and whose adjacent carbon possesses either an amino, a ketone or an hydroxyl group. After standing for forty minutes at room temperature, the reaction was stopped by the addition of 0.2 ml. of 8% stannous chloride (Mallinckrodt Analytical Reagent) in 0.3 N hydrochloric acid; 2.5 ml. of a 1% suspension of acid-washed charcoal were also added at this time. The solution was mixed and filtered. A 2 ml. aliquot of the filtrate was taken for the determination of formaldehyde according to the method developed by MacFadyen (1945). To the aliquot was added 2 ml. of 0.2% chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid, Matheson Co.) in 33 N sulfuric acid (Baker's Analyzed). The tubes were shaken and placed in a boiling water bath for 30 minutes. At the end of this time they were cooled, diluted with 4 ml. of 9 N sulfuric acid and their absorptions determined immediately in a Klett spectrophotometer using a No. 56 filter. Our standard reference compound, desoxycorticosterone, was carried through the analytical procedures from the point of taking Fraction IV to dryness onward, and all outputs of formaldehydogenic steroids are expressed in terms of weight equivalents of this compound. Recoveries averaging 85% were obtained when this compound was subjected to the entire analytical scheme.

Various other tests were conducted for the determination of steroids. The 2,4-dinitrophenylhydrazine reaction was carried out according to the directions of Gornall and MacDonald (1953). Cortisone acetate was used as a reference compound here but the results were finally expressed as weight equivalents of desoxycorticosterone. The steroids were

also taken up in absolute ethanol for absorption measurements at 240 mμ. in a Beckman spectrophotometer.

Paper chromatographic procedures were conducted in accordance with the directions of Bush (1951). We routinely used his partition system B_5 (benzene and 50% methanol, 1:1 v/v). The spots were detected under ultra-violet light after the chromatograms had been sprayed with 15% sodium hydroxide and dried under infrared lamps. Kendall's cpds. B and F were routinely run on parallel strips.

Sodium acetoacetate was prepared by hydrolyzing ethyl acetoacetate according to the directions of Ljunggren (1924) and assayed manometrically by decarboxylation in the presence of aniline citrate. All other substrates were commercial reagents. The sodium salts of acetic, isovaleric and succinic acids were employed. Control runs revealed that neither the incubation medium nor any of the substrates used nor the added ACTH preparations contributed significant amounts of formaldehydogenic materials to Fraction IV.

RESULTS

1. *Examination of various aspects of the incubation situation.* At first, heparinized rat blood was used as the incubation medium and, indeed, higher levels of steroid output were observed with it than with an artificial medium. However, the difficulties attendant upon obtaining adequate supplies of blood plus uncertainties concerning reactions between the compounds under observation and various components of blood forced us to use the artificial medium exclusively.

Using a Potter-Elvehjem homogenizer, adrenal glands were homogenized in the artificial medium and incubated. No steroid formation could be detected. Saffran and Bayliss (1953) had a similar experience with adrenal homogenates.

In one experiment, male rats of the same age and strain (12 animals) were used as a source of adrenal glands. The steroid output, under control conditions, was only approximately 60% of that obtained with female adrenals. The influences of age, sex and strain of rat upon *in vitro* steroidogenesis are but incompletely known at present and deserve further investigation.

TABLE 1. STEROID CONTENT OF THE VARIOUS CHROMATOGRAPHIC FRACTIONS

Method of determination	μg. of steroid per gm. of adrenals[1]				
	Fraction				
	I	II	III	IV	V
Production of Formaldehyde	0	7	14	265	603
Reaction with 2,4-dinitrophenylhydrazine	45	41	8	276	196
Absorption at 240 mμ.	329	267	147	312	503

[1] Weight equivalents of desoxycorticosterone.

2. *Examination of the five chromatographic fractions for steroid content.* Fractions I through V were collected in one experiment and examined as follows: 1) for content of formaldehydogenic substances, 2) for content of compounds reacting with 2,4-dinitrophenylhydrazine and 3) for absorptions at 240 mμ in the ultraviolet. (They were also chromatographed on paper, as will be discussed in the next section.) The results of these three analytical procedures are shown in Table 1. It will be seen that only Fractions IV and V contain significant amounts of formaldehydogenic substances and 2,4-dinitrophenylhydrazine reacting substances. The α,β-unsaturated ketonic structure has its maximum absorption in the ultra-violet at 240 mμ but the specificity of this measurement in the presence of other compounds of unknown structure is questionable as may be seen from the results which we obtained. Good agreement was obtained in Fraction IV between formaldehydogenicity and the formation of 2,4-dinitrophenylhydrazones. Again the specificity of the latter reaction is limited but Gornall and MacDonald (1953) report that the reaction is greatly intensified

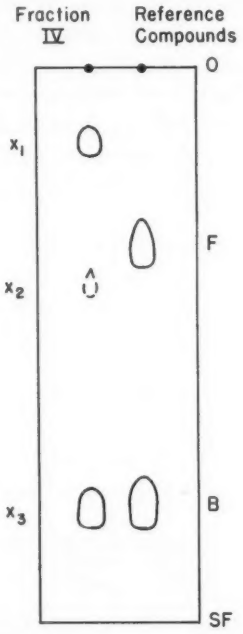

FIG. 1. A typical Paper Chromatogram of Fraction IV. *O*, starting line; *F*, 17α-hydroxycorticosterone (R_f=0.35); *B*, corticosterone (R_f=0.82); X_1, X_2, and X_3, unidentified steroids, (R_f values=0.15, 0.41 and 0.83), X_3 is most probably corticosterone; *SF*, solvent front.

with Δ^4,3-ketosteroids which possess the 17,21-dihydroxy, 20 keto configuration.

The compounds responsible for the high values found in Fraction V remain unknown. They are probably not Δ^4,3-ketosteroids, as will be seen in the next section.

3. *Examination of the in vitro adrenal steroid output by paper chromatography.* All five fractions eluted from the silica gel columns were examined by paper chromatographic methods. Recognizable steroid spots could be detected only in Fraction IV. According to Bush (1953a), a positive result, following treatment with NaOH, most probably indicates a Δ^4,3-ketosteroid.

A typical chromatogram of Fraction IV is shown in Figure 1. Unknown spot χ_3 most probably represents corticosterone, and is by far the most prominent of the three. Unknown spot χ_2 is not always seen and at best but faintly. The R_f values for spots χ_1 and χ_2 do not correspond with those of any of the other known active cortical steroids in this system although that of χ_2 is near to that of cortisone, as determined by Bush (1951). Despite its high content of formaldehydogenic compounds and those capable of forming 2,4-dinitrophenylhydrazones, Fraction V contained no steroids demonstrable by this method.

When ACTH was present in the incubation medium, an increased steroid concentration was indicated by intensification of the spots, but without alteration in the steroid pattern, when Fraction IV was chromatographed on paper.

On only one occasion, we observed a fourth steroid in Fraction IV with an R_f value similar to that of 11-dehydrocorticosterone in this system, and almost equal in intensity to the corticosterone spot. Whether this was a genuine reflection of the glandular output or an analytical artifact remains to be settled.

4. *Steroid output under control conditions.* Average pre- and post-incubation formaldehydogenic steroid contents of the flasks under control conditions are reported in Table 2. (It should be emphasized again that all formaldehydogenic steroid (FS) values are given in terms of weight equivalents of

TABLE 2. OUTPUT OF FORMALDEHYDOGENIC STEROIDS UNDER CONTROL CONDITIONS

	No. flasks	μg. FS[1] per gm. adrenals	Net output μgm. FS per gm. adrenals per 2.5 hrs.
Unincubated	12	89 (±15.1)[2]	—
Incubated	34	264 (±12.7)	175

[1] Formaldehydogenic Steroids.
[2] Standard Error of the Mean.

TABLE 3. THE EFFECT OF ACTH ON *in vitro* STEROID FORMATION

Dose ACTH units per 100 mg. of adrenals	No. flasks	Net output μg. FS per gm. adrenals per 2.5 hrs.	$\frac{x^1}{c}$	Fisher's "p"
0.1	19	200 (±14.0)	1.14	>0.05
1.0	23	171 (±18.3)	0.98	>0.05
10.0	8	317 (±23.9)	1.79	<0.01

[1] Ratio of steroid outputs in presence and absence of ACTH. Cf. Table 2 for control data.

desoxycorticosterone.) It will be seen that, on the average, 175 micrograms of FS per gram of adrenals were produced during the two and one half hour incubation period. This figure was used by us as a baseline to evaluate the effects on steroid output of the various compounds used.

5. *The effect of ACTH on in vitro steroidogenesis.* Using the Astwood ACTH, found by assay to have an average potency of 80 units per mg., we observed the responses of our glands to three dose levels of this tropic hormone. The results are shown in Table 3, and reveal our *in vitro* system to be a relatively insensitive one as far as ACTH response is concerned. Consistent stimulation was observed at the 10.0 unit dose level but results were erratic at the 1.0 unit level and only occasional enhancement of steroid output could be seen. No stimulation was ever seen at 0.1 unit level.

If ACTH which had been exposed to the effects of 10% hydrogen peroxide for twelve hours at room temperature (the peroxide later being removed by evaporation) was supplied to the glands in a dose of 10.0 units of ACTH per 100 mg. of adrenals, no stimulatory effect could be noted. However, boiling Astwood ACTH for 2 hours in 0.1 *N* NaOH only slightly decreased its potency.

TABLE 4. EFFECTS OF VARIOUS POSSIBLE HORMONE PRECURSORS ON *in vitro* STEROID FORMATION

Substrate	Final conc. in medium.	No. flasks	Net output μg. FS per gm. adrenals per 2.5 hrs.	$\frac{x^1}{c}$	Fisher's "p"
acetate	$10^{-3}M$	13	207 (±30.2)	1.18	>0.05
acetate	$10^{-2}M$	8	236 (±25.0)	1.35	<0.05
					>0.02
glycerol	$10^{-3}M$	10	264 (±67.4)	1.51	>0.05
glycerol	$10^{-2}M$	20	314 (±38.4)	1.80	<0.01
acetoacetate	$10^{-3}M$	13	301 (±38.3)	1.72	<0.01
acetoacetate	$10^{-3}M$	16	471 (±90.2)	2.69	<0.01
isovalerate	$10^{-3}M$	12	257 (±54.9)	1.47	>0.05
isovalerate	$10^{-2}M$	14	207 (±24.9)	1.18	>0.05

[1] Ratio of steroid outputs in presence and absence of substrates. Cf. Table 2 for control data.

6. *The effects of various possible hormone precursors on in vitro steroidogenesis.* Guided by the results obtained by others working in the field of lipid synthesis, we investigated the effects of four possible corticoid precursors on the rate of steroid formation. They were acetate, glycerol, acetoacetate and isovalerate. The results are given in Table 4, and show acetoacetate to be the compound most readily utilized. This preference for acetoacetate is strikingly evident even if allowance is made for the fact that it contains more carbons than either acetate or glycerol.

The fact that glycerol and acetoacetate markedly increase corticoid formation does not in itself necessarily mean that they are incorporated into steroid molecules wholly or in part. It is conceivable that they could stimulate steroid output is some indirect fashion. It was hoped therefore that more definitive results could be obtained using isotopically-labeled forms of these compounds. We could obtain only acetate (labeled with C^{14} in the carboxyl carbon) and glycerol (labeled with C^{14} in one of the alpha carbons) for incubation purposes (in a final concentration of 10^{-2} *M*). Fractions III, IV and V from the silica gel columns were collected in the case of the flasks containing the labeled substrates, evaporated almost to dryness and plated out on steel planchets for counting. The results showed that no significant amount of acetate was incorporated into any of the compounds present in these three fractions. In the case of the flasks containing the labeled glycerol, Fractions III, IV and V contained 28.9, 438.7 and 724.6 counts per minute respectively, these figures corresponding to percentage incorporations of 0.013, 0.22 and 0.36. The increases in the steroid content of Fraction IV obtained with unlabeled glycerol, at the same concentrations, indicate an incorporation of 0.81% (assuming that all of the glycerol carbons go to steroid carbons and that the average molecular weight of the steroids formed is 342). Again the high activity of Fraction V cannot be explained at present. Inconclusive results were obtained when these fractions were chromatographed on paper mainly because the low amounts of radioactivity present made localization of the active areas difficult. They did suggest, however, that a significant amount of the radioactivity was not present in Δ^4,3-ketosteroids.

Various combinations of the substrates studied above such as glycerol and acetate, glycerol and acetoacetate, etc. failed to reveal any interactions between these compounds in their effects on steroid formation. Only the effect of the more potent stimulator of steroid output was observed.

Because of its ability to increase markedly the Qo_2 of adrenal slices (Sourkes and Heneage, 1952), succinate, in a final concentration of 10^{-2} *M*, was added to the incubation flasks. No deviation from control levels of steroid output was seen.

7. *The effects of ACTH in combination with various possible hormone pre-*

TABLE 5. THE EFFECTS OF ACTH AND VARIOUS POSSIBLE HORMONE PRECURSORS ON *in vitro* STEROID FORMATION

Additions	No. flasks	Net output μg. FS per gm. adrenals per 2.5 hrs.	$\frac{x^1}{c}$	Fisher's "p"
ACTH[2]	23	171 (±18.3)	0.98	>0.05
ACTH+acetate[3]	7	199 (±33.3)	1.14	>0.05
ACTH+glycerol	8	146 (±20.1)	0.83	>0.05
ACTH+acetoacetate	11	201 (±24.3)	1.15	>0.05
ACTH+isovalerate	10	107 (±20.0)	0.61	<0.01

[1] Ratio of steroid outputs in the presence and absence of the additions. Cf. Table 2 for control data.
[2] ACTH doses were all 1.0 unit per 100 mg. of adrenals.
[3] All precursors were present in the incubation flasks in a final concentration of $10^{-2}M$.

cursors. Despite the unpredictable responses obtained with the use of 1.0 unit of ACTH per 100 mg. of adrenals, it was decided to see what effect the addition of ACTH at that dose level would have when one of the above-mentioned substrates was also present in the incubation vessel. The results are given in Table 5. It can be seen that these combinations produced steroid outputs essentially like those of the control situation, except in the case of isovalerate and ACTH where the output was significantly less than that of the controls. The stimulatory effects of the substrates appear to have been blocked by the presence of ACTH.

8. *Preliminary observations on in vitro steroidogenesis in hypophysectomized rats.* To evaluate the effects of pituitary removal on the steroidogenic

TABLE 6. STEROID OUTPUTS BY ADRENAL GLANDS FROM HYPOPHYSECTOMIZED RATS

	No. flasks	Steroid content of flasks μg. FS per g. adrenals
Intact Adrenals	34	264 (±12.7)
Hypox. Adrenals	6	148 (±19.7)
Hypox. Adrenals plus ACTH[1]	3	329 (±95.4)

[1] ACTH dose was 10.0 units per 100 mg. adrenals.

capabilities of rat adrenal glands, we incubated adrenals from rats which had been hypophysectomized for two to three weeks with and without ACTH. The results are shown in Table 6. Since we have no values for the pre-incubation steroid contents in this situation, strict quantitative comparisons cannot be drawn between these findings and those obtained with glands from intact rats. Two conclusions may be tentatively drawn, however. First, on a weight basis, adrenals from hypophysectomized rats are not capable of synthesizing corticoids at a rate comparable to that seen with glands from intact rats; and second, adrenals from hypophysecto-

mized rats are capable of responding to the stimulatory effects of ACTH with little or no delay.

DISCUSSION

In an *in vitro* system such as ours, only limited significance can be attributed to negative results, i.e., those in which a compound causes neither a marked enhancement nor depression of steroid output. Such factors as cellular permeability, amount of substrate provided and amount of substrate already present in the glands must be evaluated before valid conclusions can be drawn. The optimal conditions for the study of *in vitro* steroidogenesis have yet to be achieved. Perhaps achievement of these optimal conditions will act to decrease the wide variations in steroid output in duplicate determinations so frequently encountered by workers in this field.

The finding that corticosterone is the principal steroid formed by rat adrenal glands *in vitro* is in good agreement with the findings of others. Bush (1953b) could detect only this steroid, in significant quantities, in rat adrenal venous blood, and Done *et al.* (1952) found only negligible amounts of 17-hydroxy corticosteroids in the blood of the rat. The steroid produced by the rat which is more polar than Kendall's compound F (Spot X_1 in Fig. 1) is an intriguing one and work is currently under way in an attempt to identify it. Its R_f value is quite similar to that of one component of Wintersteiner's "amorphous fraction," as determined by Bush (1951). Since it occurs near the origin of the chromatogram, however, the possibility exists that this spot represents the incomplete separation of two or more steroids, and further investigations of it are currently underway. Haynes *et al.* (1953) were also able to detect compounds more polar than hydrocortisone when this compound was incubated with beef adrenal glands.

The compound or compounds responsible for the high degree of chemical reactivity in Fraction V (the methanol eluate) obtained from the silica gel columns are not known. They are formaldehydogenic, absorb strongly at 240 mμ in the ultra-violet, are capable of forming 2,4-dinitrophenylhydrazones, and are capable of incorporating glycerol wholly or in part into their structures (through obviously all of the compounds present may not possess all of these properties), and yet, do not seen to be Δ^4,3-ketosteroids as determined by paper chromatography. Further studies on this fraction, employing both chemical and biological procedures, do seem to be warranted by the presently available data.

Our attempts to demonstrate an *in vitro* stimulatory effect of ACTH have been unsatisfactory from the point of the amounts of hormone required. The responses obtained with 1.0 unit per 100 mg. of adrenals were

erratic and are attributed at present to our ignorance concerning the optimal conditions required for the demonstration of an ACTH effect. Saffran and Bayliss (1953) have suggested that the *in vitro* stimulatory effect of ACTH on steroid output by rat adrenals be employed as a means of assaying this tropic hormone. Our findings that compounds other than ACTH (e.g. acetoacetate) can markedly enhance steroid output would seem to decrease the validity of such an assay method.

The finding that acetoacetate can cause significant increases in adrenal steroid formation is partial confirmation of the findings of Brady and Gurin (1951) that rat liver slices readily incorporate acetoacetate into cholesterol and that this is done without the prior formation of 2-carbon fragments. Our results do not, however, confirm those of the above authors and those of Zabin and Bloch (1950) concerning the utilization of isovalerate for cholesterol synthesis. These discrepancies may reflect differences in synthetic pathways for the formation of cholesterol on one hand and adrenal steroids on the other. We cannot explain the relative failure of acetate to stimulate steroid output under our conditions. Perhaps an adequate source of 2-carbon fragments already exists in the gland and the material supplied is superfluous. The apparent neutralization of the stimulatory effects of certain substrates, notably acetoacetate and glycerol, by ACTH is not easily understandable. Some have speculated that perhaps more than one synthetic pathway exists in the adrenal for the formation of corticoids, or that ACTH exerts its stimulatory effect by accelerating one or more rate-limiting reactions involved in the synthesis of adrenal steroids. Such a situation might account for these findings with competition for substrates and energy sources playing a major role.

The preliminary observations on steroid output by adrenal glands from hypophysectomized rats are of interest in two respects. First, as in the case with intact rats, adequate starting materials for steroid formation are already present in the gland and no added substrate is required. What fraction this output is of the maximal output possible is not known. Second, no preliminary "conditioning" period is necessary to enable hypophyseoprivic adrenals to respond to ACTH with an increased output of corticoids. They respond almost immediately as was evident in the marked increases which were obtained in our relatively short incubation periods.

SUMMARY

Procedures are described for the incubation of quartered rat adrenal glands and for the extraction and purification of the steroids thus produced *in vitro*. The corticoids formed were measured as formaldehydogenic steroids. The output, under control conditions, was 175 micrograms of formaldehydogenic steroids per gram of adrenals per $2\frac{1}{2}$ hours.

Paper chromatographic analyses of the corticoid output revealed that corticosterone was the predominant steroid formed *in vitro* by rat adrenal glands. In addition, at least two Δ^4,3-ketosteroids of unknown structure were observed.

An *in vitro* stimulatory effect of ACTH on steroid output could be demonstrated, but relatively large amounts were required for reproducible results.

Acetate, glycerol, acetoacetate and isovalerate were studied as possible hormone precursors. Only acetoacetate and glycerol markedly enhanced the rate of *in vitro* steroid formation. This stimulatory effect was totally abolished if both the substrate and ACTH were supplied to the glands simultaneously.

Preliminary observations reveal that, in adrenal glands from hypophysectomized rats, steroid output is markedly reduced, but that they are capable of responding to ACTH with an increased corticoid output without appreciable delay.

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THE EFFECT OF ADRENAL CORTICAL HORMONES ON THE ANAEROBIC GLYCOLYSIS AND HEXOKINASE ACTIVITY[1]

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THE relation between the adrenal cortical hormones and carbohydrate metabolism has been known for a long time (see Britton's review, 1930). The low blood sugar levels in patients with Addison's disease, discovered by Porges (1909), were also observed in adrenalectomized animals (Artundo, 1927), where there was found a decreased liver glycogen (Houssay and Artundo, 1929, Cori and Cori, 1929). Moreover, the level of glycosuria in partially depancreatized rats decreased after adrenalectomy (Long *et al.*, 1940). A demonstration that these effects were due to the action of adrenal hormones was given later. Ingle (1941) produced glycosuria in force-fed normal rats by the administration of cortisone, corticosterone, and 17-hydroxycorticosterone; and Lazarow and Berman (1950) with cortisone acetate. Increased deposition of glycogen in the liver by the administration of cortical hormones was also observed (Grattan and Jensen, 1940; Long, 1942). In muscle, however, glycogen deposition was not affected (Cori and Cori, 1929; Verzar, 1952). On the contrary, in *in vitro* experiments with rat diaphragm it was found that corticosteroids inhibited glycogen formation (Verzar and Wenner, 1948; Bartlett *et al.*, 1949), as well as glucose uptake (Leupin and Verzar, 1950). With mouse diaphragm, Dirscherl and Brener (1951) found that desoxycorticosterone acetate inhibited the anaerobic glycolysis when the glycolysis values were high. The mechanism of action of the adrenal cortical hormones on the regulation of carbohydrate metabolism remains unknown. Long *et al.* (1940) considered this effect a consequence of "the stimulation of protein metabolism," a hypothesis which was contradicted by Verzar (1952), who postulated that the main action of the adrenal cortical hormones is the regulation of glycogen metabolism. In preceding papers (Kit and Barron, 1952; and

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Kit *et al.*, 1953) it was shown that adrenal cortical hormones added to suspensions of lymphatic cells, in amounts which had no effect on their respiration, inhibited synthesis of protein and nucleic acids. We present here experiments on the effect of these hormones on the anaerobic glycolysis of normal and tumor lymphatic cell suspensions, red cells, yeast, and mouse diaphragm. Glycolysis was inhibited in some of these cells and in diaphragm, whereas the glycolysis of tumor cells and of yeast was not affected. The inhibition of glycolysis found in some of these tissues seems to be due to the inhibition of hexokinase, since the hexokinase from the tumor cells and from yeast was not affected by the corticosteroids.

EXPERIMENTAL

The white mice used for the anaerobic glycolysis were Swiss white mice, Webster strain, from 15 to 20 gm. in weight. In all cases a half-diaphragm was taken per Warburg vessel. The tissue was suspended in 2 ml. of Ringer-bicarbonate containing 0.01 *M* glucose (unless otherwise indicated) and saturated with N_2:CO_2 (95:5) with a pH value of 7.4 at 38° C. The suspensions of lymphatic cells from rabbit appendix and of mouse lymphosarcoma (Gardner's lymphosarcoma in C3H brown mice from the Jackson Laboratories, Bar Harbor, Maine) were prepared according to Kit and Barron (1952). Mice with Erhlich's ascites tumor were donated by Dr. Williams-Aschman of this University. The red cells were washed twice with Ringer-bicarbonate solution; the baker's yeast cells were washed five times with distilled water, and both were suspended in Ringer-bicarbonate-glucose. Compound F (17-hydroxycorticosterone), compound E (17-hydroxy-11-dehydrocorticosterone), and compound E acetate were kindly provided by Merck and Co.; desoxycorticosterone, by Organon, Inc.; and compound S (Δ^4-pregnene-17-β-21-diol-3,20-dione), by Dr. Bergenstal of this University. The corticosteroids were dissolved in methyl alcohol, and the alcohol was evaporated off from the Warburg vessels by evacuation. Control vessels with evaporated methyl alcohol without hormone gave similar values to those without alcohol. Glutathione was obtained from Schwartz and Co., New York, and potassium adenosinetriphosphate (KATP) and yeast hexokinase from Pabst and Co., Milwaukee, Wisconsin. Animal hexokinase was prepared as follows: The tissue was immediately cooled to 0° C.; suspended (5:1) in a solution containing 36 ml. of 0.154 *M* KCl, 10 ml. of 0.154 *M* $KHCO_3$, and 4 ml. of 0.154 *M* $MgCl_2$ saturated with N_2:CO_2 (95:5) and cooled to 0° C. It was homogenized in a glass homogenizer, and after filtration through gauze it was centrifuged at 0° C. for 10 minutes at 600 g. The supernatant fluid was centrifuged *in vacuo* in a Spinco Model L refrigerated centrifuge for 30 minutes at 152,000 g. Three distinct phases were found in the tubes: the upper, lipoid-cloudy

phase; the clear middle phase; and the bottom precipitate. The lipoid phase, as well as the precipitate, was discarded and the middle, clear solution was used as the source for the enzyme. Enzyme activity was measured manometrically. Each vessel contained in the main compartment 1 ml. of the enzyme solution with varying amounts of protein (final concentration of 1 to 4 mg. per ml.), and 0.2 ml. of 0.2 *M* KF, 0.1 ml. of 0.154 *M* $KHCO_3$, 20 μM of KATP in 0.2 ml., and 0.4 ml. of KCl-bicarbonate buffer pH 7.4. The side arm contained 0.1 ml. of 0.1 M glucose. Solution and gas phase of vessels were saturated with N_2:CO_2 (95:5), and the reaction was measured at 26° C. unless otherwise indicated. The hexokinase obtained by fractional centrifugation was very active, and the blank values (CO_2 formation in the absence of glucose, probably due to hydrolysis of ATP) were low (around 5 cmm./hour/4 mg. of protein per ml.).

The spectrophotometric measurements were made in quartz cells with the DU Beckman spectrophotometer. Glutathione was determined according to Grunert and Phillips (1951), the readings being made at 5200 A and at 7° C. Under these conditions the color remained stable for at least 10 minutes.

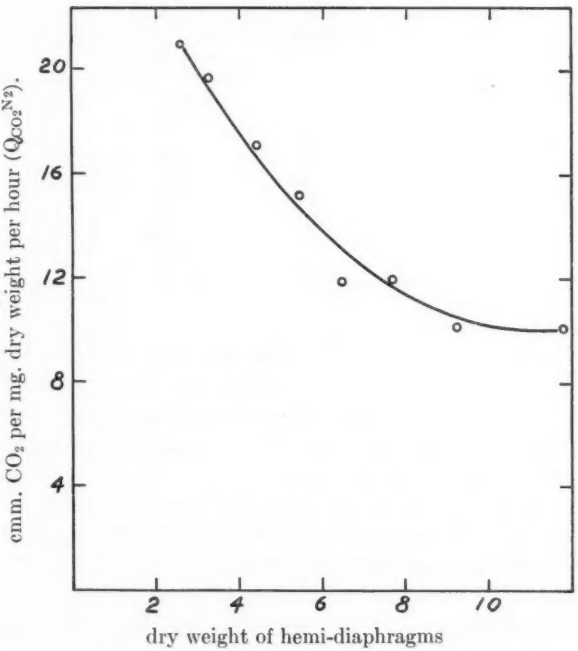

FIG. 1. The relation between anaerobic glycolysis of mouse diaphragm and dry weight.

Anaerobic Glycolysis of Mouse Diaphragm and of Lymphatic Cells.—Dirscherl and Knuchel (1950) found that the anaerobic glycolysis of mouse diaphragm varied with the age of the animal, being high in young mice ($Q_{CO_2}^{N_2}$, 7.1) and low in old mice ($Q_{CO_2}^{N_2}$, 3). We have essentially confirmed these findings, although the values obtained were higher. Anaerobic glycolysis, high in thin diaphragms (4.2 mg. dry weight), diminished as the weight increased up to 13 mg. dry weight, where it remained constant (Fig. 1). The average weight of the hemidiaphragms used in the experiments with adrenal corticosteroids was about 5 mg. In every case one hemidiaphragm was the control, while the other contained the adrenal hormone.

The lymphatic cell suspensions had a very active anaerobic glycolysis. The $Q_{CO_2}^{N_2}$ (cmm. CO_2 produced per mg. dry weight per hour) of lymphatic cells of rabbit appendix was 25.1, a value 2.5 times as high as that obtained

TABLE 1. EFFECT OF COMPOUND F ON ANAEROBIC GLYCLOYSIS

Cells suspended in Ringer-bicarbonate saturated with N_2:CO_2 pH 7.4. Glucose 0.01 *M*. All experiments, except those of yeast were run at 38° C. Yeast, 26° C. Figures given are averages of three to four different experiments, each experiment in triplicate. The figures marked with an asterisk are cmm. per mg. dry weight of tissue.

Tissue	Comp. F μg./ml.	CO_2 output, c.mm.		Incubation in minutes
		Control	Comp. F	
Ascitis tumor (mouse)	100	1,000	980	120
Human red cells	102	136	115	120
Yeast	100	709	682	120
Rabbit red cells	100	232	221.5	120
Lymphatic cells (rabbit appendix)	40	156	141	120
Lymphatic cells (rabbit appendix)	50	96	85	60
Lymphatic cells (rabbit appendix)	100	96	58	60
Gardner's lymphosarcoma (mouse)	100	516.5	511	120
Gardner's lymphosarcoms (mouse)	120	255	253	120
Diaphragm (mouse)	50	24.8*	13.6*	120
Diaphragm (mouse) (0.1 *M* glucose)	150	20.3*	24.7*	120

in experiments with the mucosa of the appendix (Redfield and Barron, 1950). The $Q_{CO_2}^{N_2}$ of the cells of Gardner's mouse lymphosarcoma was 53.2, also higher than the values reported by Summerson *et al.* (1948). The $Q_{CO_2}^{N_2}$ of Ehrlich's mouse ascites tumor was 48.2, a value in agreement with that found by Warburg and Hiepler (1952).

The anaerobic glycolysis of the appendix lymphatic cells was inhibited 12% by 50 μg./ml. of compound F, and 40% by 100 μg. (2.8×10^{-4} *M*). On the other hand, the anaerobic glycolysis of the cells from ascites tumor and from lymphosarcoma were not affected at all by 100 μg./ml. Equally resistant were human and rabbit red cells and bakers' yeast cells. The glycolysis of mouse diaphragm was inhibited 45% with 50 μg. of compound F per ml., in experiments of two hours duration. Surprisingly, this inhibi-

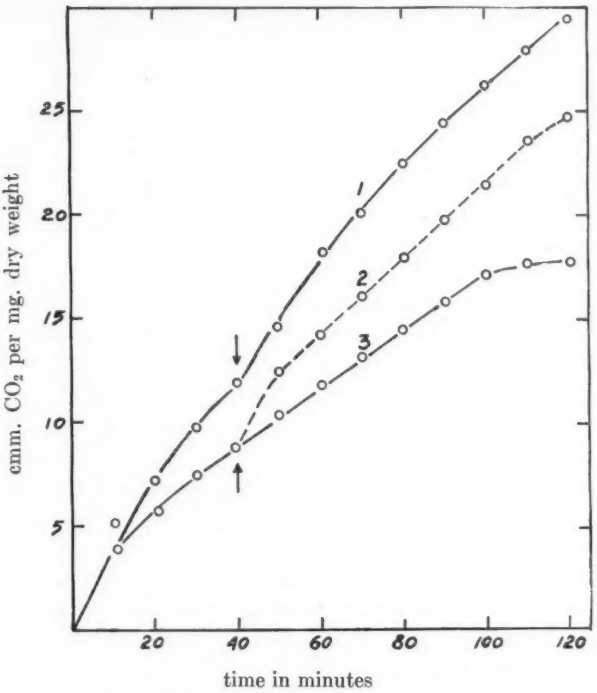

FIG. 2. Reversal by glucose of the inhibition of glycolysis by compound F (50 μg./ml.). Mouse Diaphram. Experiment started in the presence of 0.01 *M* glucose. At time indicated by arrow (40 minutes) 0.1 *M* sugar was added to 1 and 2. 1. Control; 2. Compound F with glucose added; 3. Compound F no glucose added.

tion did not occur when the glucose concentration was increased to 0.1 *M* (Table 1). High glucose concentrations not only protected the tissue glycolysis against inhibition by compound F but also released the inhibition when added 40 minutes after the glycolysis measurements started, i.e., one hour after the tissue was in contact with the hormone (Fig. 2).

The inhibition of glycolysis produced by compound F was roughly proportional to the concentration of the hormone. With 50 micrograms per ml. of fluid it increased with the time of incubation, whereas with 150 micrograms per ml. inhibition reached its maximum value at the end of one hour and remained the same for the following hour (Table 2).

Glycolysis inhibition was observed on addition of other corticosteroids, such as compound E, compound E acetate, compound S and desoxycorticosterone, there being no striking difference in the degree of inhibition (Table 3).

TABLE 2. THE EFFECT OF HORMONE CONCENTRATION (COMPOUND F) ON THE ANAEROBIC GLYCOLYSIS OF MOUSE DIAPHRAGM*

Compound F μg./ml.	CO_2 formation in c. mm.	
	60 min.	120 min.
None	13.52	24.8
50	9.05	13.6
100	7.2	11.0
150	4.3	7.8

* The values given are per mg. dry weight.

The Effect of Glutathione on the Inhibition of Glycolysis by Compound F.— Although it is known that cysteine and other -SH compounds combine with glucose (Shubert, 1939), the probable protecting power of glutathione against inhibition of glycolysis by compound F was tested. In these experiments neutralized glutathione (0.01 *M*) was added to the Ringer-bicarbonate-glucose solution and was saturated with N_2:CO_2. Anerobic glycolysis of the mouse hemidiaphragms was measured in these solutions with and without 50 μg. of compound F per ml. of solution. The glycolysis values in the presence of glutathione were very erratic. It seems justifiable, however, to compare these values with those obtained in the presence of compound F (Table 4). With the exception of one experiment (No. 2), the presence of glutathione protected the diaphragm from inhibition of anaerobic glycolysis by compound F.

Disappearance of Compound F from Solutions with Mouse Diaphragm.— It has been reported by Schneider and Horstmann (1952) that the adrenal corticosteroids are readily metabolized by the liver and kidney but not by skeletal muscle. When compound F was incubated with mouse diaphragm at 26° C., disappearance of compound F from the medium containing the muscle occurred slowly and steadily up to 20 minutes, the rate of disappearance increasing with the time of incubation (Table 5). In these experiments the compound F remaining in the solution was measured spectrophotometrically after centrifugation for 15 minutes at 3,000 rpm. in the Inter-

TABLE 3. INHIBITION OF THE ANAEROBIC GLYCOLYSIS OF MOUSE DIAPHRAGM BY CORTICOSTEROID HORMONES

Hormone	Amount μg./ml.	Inhibition, per cent	
		60 min.	120 min.
Compound F	50	33	45
Compound F	100	47	55
Compound E	100	55	68
Compound E Acetate	50	32	35
Compound S	50	23	41
Desoxycorticosterone	50	52	59
Desoxycorticosterone	100	50	65

TABLE 4. EFFECT OF GLUTATHIONE ON THE INHIBITION BY COMPOUND F OF ANAEROBIC GLYCOLYSIS (MOUSE DIAPHRAGM)

Ringer-bicarbonate containing 0.01 *M* glucose and glutathione, saturated with N_2:CO_2 (95:5), 2 ml. per vessel. Compound F, 50 μg. per ml. Figures given are cmm. CO_2 per mg. dry weight per hour.

Exp. No.	CO_2 formation	
	Control	Compound F
1	9.43	9.35
2	16.50	10.0
3	8.60	8.25
4	10.10	9.30
5	5.64	4.40
6	5.90	6.34
7	5.80	6.15
8	8.40	7.70

national Refrigerated Centrifuge, Model PR1. The amount of compound F which disappeared was calculated by subtracting the optical density at 2450 A of the fluid containing diaphragm and compound F from the optical density of the fluid and diaphragm alone (Fig. 3). The amount of compound F which disappeared in 1 gram of fresh muscle was calculated from these data as 52.2 μg.

The Inhibition of Hexokinase by Corticosteroid Hormones.—The inhibition of glycolysis by the adrenal corticosteroids must be due either to interference with the penetration of glucose into the cells or to the inhibition of glycolytic enzymes up to the formation of acid, since the manometric method used in the experiments revealed inhibition of acid formation. It was thought that hexokinase may be the enzyme directly connected with inhibition of glycolysis, since glucose penetration into the muscle requires its phosphorylation by this enzyme. The activity of hexokinase from skeletal muscle (rat) was inhibited at the end of one hour 20% with 50 μg. of compound F per ml. of fluid, and 50% with 100 μg. in the experiment plotted in Figure 4. Inhibition of hexokinase was also observed on addition of the other corticosteroids. With 100 micrograms per ml., compound F in-

TABLE 5. PENETRATION OF COMPOUND F INTO THE MOUSE DIAPHRAGM

One-half diaphragm incubated at 26° C. in Ringer-bicarbonate saturated with N_2:CO_2 containing 0.01 M glucose and 100 μg. of compound F per ml.—Δ2450 Å values, are the difference of optical density between the solution containing compound F and the solution with compound F and diaphragm. The optical density of the solution containing diaphragm alone was subtracted in every case.

Time of incubation, minutes	−Δ2450 Å	Weight of diaphragm mg. (dry weight)
5	0.010	4.4
10	0.030	3.3
20	0.047	2.4
30	0.048	4.0

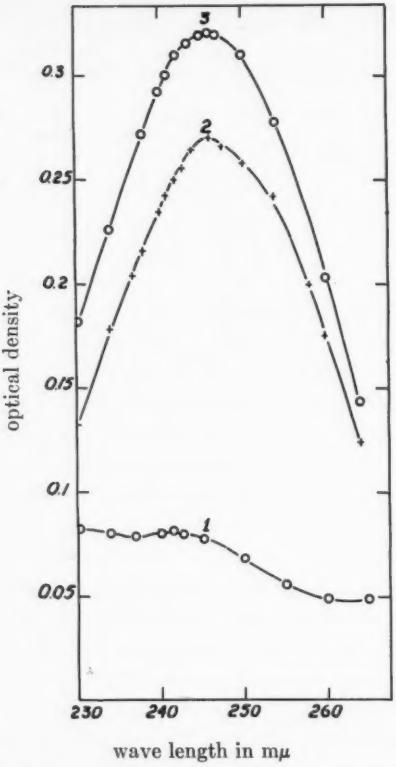

FIG. 3. Disappearance of compound F from solution containing mouse diaphragm, as measured 20 minutes after addition of hormone. 1.—Absorption spectrum of buffer containing diaphragm and no compound F.2.—Absorption spectrum of buffer containing diaphragm plus compound F (values of 1 have been subtracted). 3.—Absorption spectrum of buffer containing 100 μg. compound F per ml.

hibited 45%; compound E, compound S and desoxycorticosterone, about 30%. There was a relation between the degree of inhibition and the concentration of the hormone (Table 6).

Inhibition of hexokinase from skeletal muscle by compound F was also found in the hexokinase from heart muscle (rat) and from the lymphatic cells of rabbit appendix. Hexokinase from Gardner's mouse lymphosarcoma, from mouse ascites tumor, from brain, and from yeast was not inhibited on addition of 100 μg. of compound F per ml. (Table 7).

Compound F and Glutathione. The inhibition of hexokinase by the corticosteroid hormones could be due to their combination with the -SH groups in the protein moiety which are essential for enzyme activity. The presence

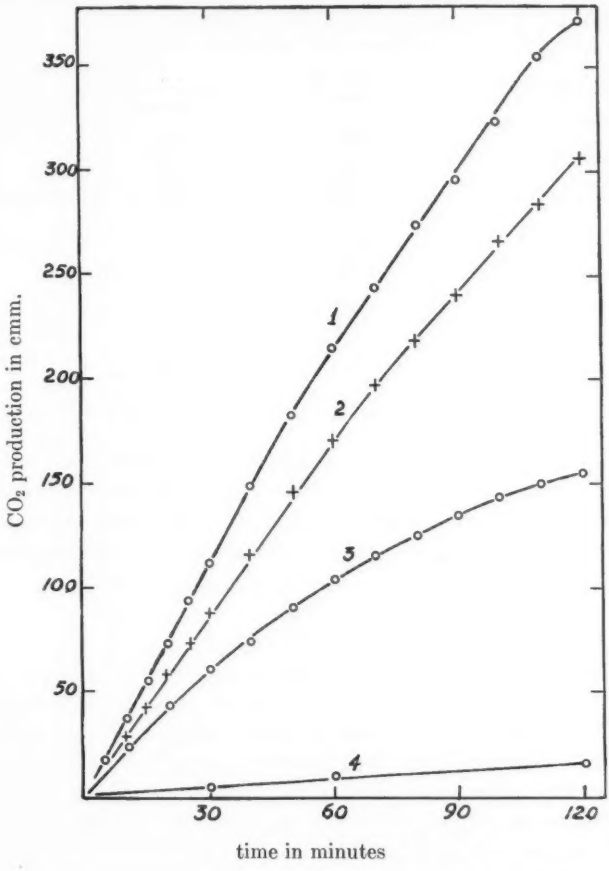

FIG. 4. Effect of compound F on the activity of muscle hexokinase (rat). Activity measured manometrically. Temp. 26° C. Protein, 4.3 mg./ml.; 1. Control; 2. Compound F 50 μg. per ml.; 3. Compound F. 100 μg. per ml.; 4. Enzyme with no glucose

of carbonyl groups in the corticosteroids and the ready combination of these groups with -SH groups make plausible the hypothesis that inhibition is produced by combination of the -SH groups of hexokinase with the corticosteroids. To test this possibility 3 ml. of a glutathione solution in oxygen-free 0.01 *M* phosphate pH 7, containing 300 micrograms (3.26×10^{-4} *M*) was mixed with 5 ml. of a compound F solution in the same buffer containing 500 μg. (2.78×10^{-4} *M*). An aliquot of this mixture as well as of the compound F and glutathione solutions was taken for spectrophotometric measurements (0.3 ml. diluted to 3 ml.). As can be seen in Figure 5, addi-

TABLE 6. EFFECT OF ADRENAL CORTICOSTEROIDS ON RAT MUSCLE HEXOKINASE ACTIVITY*

Corticosteroid	Amount μg./ml.	CO_2 production c.mm.	
		Control	Hormone
Compound F	100	184	100
Compound F	50	82	57.2
Compound F	25	140	116
Compound F	12.5	153.5	149.5
Compound E	100	118	80.8
Compound E	50	55.4	39
Compound E	25	55	49.5
Compound S	100	80	56
Compound S	50	80	75
Desoxycorticosterone	100	92	63.6

* Enzyme activity determined manometrically. Incubation time, 60 min.

tion of glutathione to compound F reduced the optical density of compound F at 246 mμ by 23%. When the free glutathione remaining in the mixture was determined colorimetrically, it was found that 29% of the added glutathione was combined. The compound thus formed has not yet been isolated, nor the stoichiometry determined accurately.

DISCUSSION

The experiments presented in this paper on the inhibition by the corticosteroid hormones of the anaerobic glycolysis of diaphragm are in agreement with the findings of the large number of investigators who observed *in vivo* low blood sugar levels in adrenalectomized animals and glycosuria in normal animals after administration of corticosteroids, and of those who observed *in vitro* decreased glucose utilization and glycogen formation on addition of corticosteroids to rat diaphragm.

Inhibition of glycolysis was also observed in the normal lymphatic cells

TABLE 7. EFFECT OF COMPOUND F ON THE ACTIVITY OF HEXOKINASE

1 ml. of enzyme solution: 0.2 ml. of 0.2 *M* KF; 0.1 ml. of 0.154 *M* $KHCO_3$; 0.2 ml. KATP containing 20μ*M*; 0.1 ml. of 0.1 *M* glucose; 0.4 ml. of KCl-$KHCO_3$ buffer, gas phase N_2:CO_2, pH 7.4 Temp. 26° C. Time of incubation, 120 min.

Source of enzyme	Amount of Compound F μg. per ml.	CO_2 production, cmm.		Time of Incubation
		Control	Hormone	
Skeletal muscle (rat)	100	273	172.5	60
Heart muscle (rat)	100	91	71.4	60
Lymphatic cells (rabbit appendix)	100	55	40	60
Gardner's lymphosarcoma (mouse)	100	90	95.5	120
Ascitis tumor (mouse) (38° C.)	100	194.1	192.2	120
Brain (rabbit)	50	87.5	82	120
Yeast	150	207	199.2	70

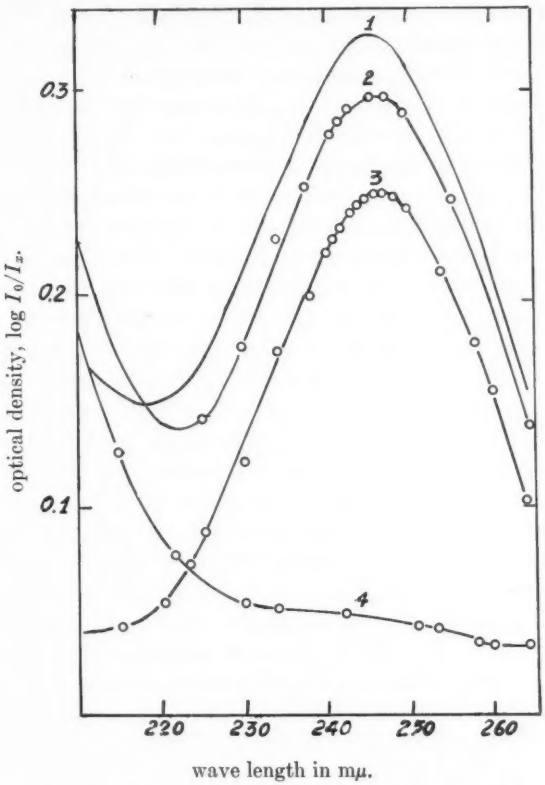

FIG. 5. The combination of compound F with glutathione as observed spectrophotometrically. In 0.01 *M* phosphate, pH 7.0. 1. Compound F; 2. Compound F+Glutathione; 3. Absorption spectrum of the complex, calculated by subtracting 4 from 1; 4. Glutathione.

of the rabbit appendix, although the glycolysis of tumor lymphatic cells (mouse lymphosarcoma, and Ehrlich's ascites tumor), of red cells, and of yeast was not affected. These differences were duplicated when the experiments were performed in the hexokinase present in the supernatant fraction from these tissues. The hexokinase from tissues and cells where glycolysis was inhibited by the adrenal hormones (diaphragm, normal lymphatic cells) was inhibited, whereas the hexokinase from cells where glycolysis was not affected (tumor lymphatic cells, yeast) remained unaffected. The correlation between inhibition of glycolysis and of hexokinase may be explained by postulating that the adrenal cortical hormones inhibit glycolysis by inhibition of hexokinase, the enzyme which phosphorylates glu-

cose and thus initiates glucose metabolism in these tissues. Hexokinases are -SH enzymes, i.e., they require the presence of -SH groups for activity (Barron, 1951); and it is possible that the adrenal cortical hormones here employed combine with the -SH groups of the enzyme and thus produce inactivation. Evidence for this assumption was found in the decrease of absorption spectrum of compound F on addition of glutathione and in the decrease of free glutathione (as measured by the colorimetric determination of the -SH groups) left in these solutions. The protection against glycolysis inhibition produced by high concentrations of glucose may be due to the attachment of glucose to the -SH groups of the enzyme which thus forms the substrate-enzyme complex. The ready combination of glucose with cysteine in neutral solutions and at room temperature is known (Schubert, 1939). There would be, thus, competition for the -SH groups of the enzyme between glucose and the corticosteroids. This inhibition is different from that produced by the adrenal cortical extracts used by Colowick *et al.* (1947), who reported that inhibition of hexokinase by adrenal cortical extracts in diabetic muscle required the presence of pituitary extracts. Hexokinase inhibition under such conditions was, according to them, released by insulin. The lack of hexokinase inhibition in tumor lymphatic cells, in red cells, in brain, and in yeast may be due to the different spatial distribution of the -SH groups in the hexokinase of these cells or to the presence of side groups vicinal to the -SH groups which produce steric hindrances; in both cases the result would be less reactivity of the -SH groups. It is known that the hexokinases from yeast, brain, liver and muscle is different in each case as shown by the different behavior toward certain agents (Slein *et al.*, 1950; Weil-Malherbe and Bone, 1951). Brain hexokinase, for example, is rapidly destroyed by treatment with butanol, whereas yeast hexokinase is not affected by it. Muscle hexokinase is inhibited by glucose-6-phosphate, whereas yeast hexokinase is not.

SUMMARY

The anaerobic glycolysis of mouse diaphragm varied with the weight of the tissues, being high in thin diaphragms, low in heavy-thick diaphragms. With tissues from 50 to 80 mg. fresh weight, it was 13.5 cmm. CO_2 per mg. dry weight ($Q^{N_2}_{CO_2}$). The $Q^{N_2}_{CO_2}$ value of lymphatic cells suspensions from rabbit appendix was 25.1; from Gardner's mouse lymphosarcoma, 53.2; and from Ehrlich's mouse ascites tumor, 48.2.

The anaerobic glycolysis of lymphatic cells from the appendix and from the mouse diaphragm was inhibited on addition of compounds F, E, E acetate, and S, and of desoxycorticosterone, whereas the anaerobic glycolysis of tumor lymphatic cells, of yeast, and of red cells was not affected. High concentrations of glucose protected against inhibition and released

the inhibition when added one hour after the addition of hormone. Addition of glutathione had also protective effect. Hexokinase prepared from skeletal muscle and from normal lymphatic cells was inhibited by the corticosteroids, whereas hexokinase from yeast and from lymphosarcoma was not affected.

On addition of neutralized glutathione to a solution of compound F, there was a decrease in the absorption spectrum of compound F and a decrease in the amount of -SH groups in glutathione, an indication of compound formation. It is postulated that adrenal cortical hormones inhibit glycolysis by combination with the -SH groups of hexokinase.

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THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 18, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Hotel reservations may be made through the San Francisco Convention and Visitors' Bureau, Room 300, Civic Auditorium, San Francisco, California. They should be accompanied by the official application form which has appeared weekly in the J.A.M.A. since December 1953.

Arrangements have been made for members of this society who do not belong to the AMA to be assigned rooms during the days of the meeting of the Endocrine Society. Applications from nonmembers of the AMA should specify dates of arrival and departure and be accompanied by a deposit of $10.00 per room.

Confirmations will not be sent until after February 1, 1954, but *applications should be made promptly* in order to insure the reservation.